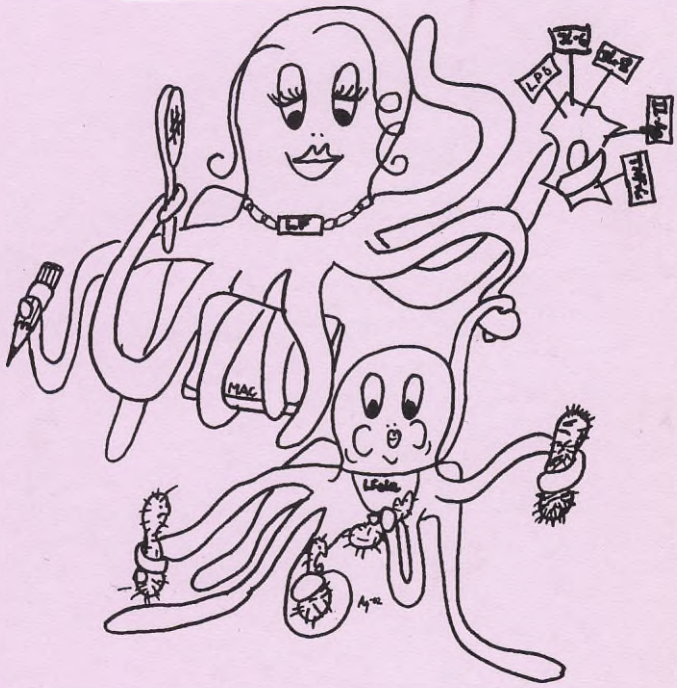
Anti-infectious and anti-inflammatory activities of lactoferrin and fragments thereof

Liliana Håversen



Departments of Clinical Immunology and Clinical Bacteriology
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Liliana Håversen

Fakultetsopponent:
Professor Arne Forsgren
Avdelning för Medicinsk Mikrobiologi
Universitetssjukhuset MAS Malmö

Avhandlingen baserar sig på följande arbeten:

I. **Håversen, L, Engberg I, Baltzer L, Dolphin G, Hanson L Å, and Mattsby-Baltzer I.**
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Abstract

Lactoferrin (LF), a major protein present in milk, mucosal secretions and secondary granules of neutrophils, has been suggested to participate in host defense at mucosal surfaces and to mediate anti-inflammatory activities. A pepsin-derived fragment of LF has been shown to contain the antibacterial domain of the protein. Despite this proposed important dual capacity of LF at the mucosal membranes there are few *in vivo* studies to support these effects. Our aim was to investigate if anti-infectious or anti-inflammatory activities on mucosal surfaces could be mediated by perorally given LF or synthetic fragments of the antibacterial region of LF in experimental animal models, to gain insight into how LF mediates the anti-inflammatory activities *in vitro*, and to define the sequence in the antibacterial region of the LF molecule responsible for the antimicrobial activity.

Experimental mouse models of urinary tract infection (UTI) induced by *E.coli* O6K5 and dextran sulphate (DX)-induced acute colitis were used. The number of *E.coli* present in the urinary tract and the urinary and systemic inflammatory response (urinary leukocytes, urinary and systemic IL-6 levels) in mice with UTI were reduced by the LF treatment compared to the control group. Mice treated with LF peptide fragments (HLD1 and 2) also showed reduced numbers of bacteria in the kidneys. The perorally given LF was found to pass over to the circulation and urine. HLD2 mediated significant bactericidal activity against *E.coli* when tested *in vitro* in mouse urine. The damaging effects induced by DX exposure (presence of blood in the faeces, colon shortening, IL-1β serum levels, crypt score) were delayed or reduced in mice treated with LF or the peptides. The number of inflammatory cells present in the colon after 7 days of DX exposure (F4/80 macrophages, CD4- and TNF-α–positive cells) was lower in the LF treated group compared to the control. LF also reduced shortening of the colon when given orally to animals with an already established inflammatory response as induced by two days of DX exposure. LF was shown to down-regulate the secretion of TNF-α, IL-1β, IL-6, IL-8 and IL-10 in monocytic cell lines (THP-1, Mono Mac 6) stimulated with LPS. The down regulation of the cytokines was reflected at the transcriptional level. Thus LPS-induced TNF-α-, IL-1β-, IL-6-, and IL-8 mRNA as shown by reversed transcription PCR were reduced as well. The known binding of LF to LPS could not explain the reduced cytokine mRNA expression and protein secretion since the effects were observed also when LF was added 30 min after the LPS to the cell assay. In addition, also IL-1β induced IL-6 secretion was down-regulated in the presence of LF. Moreover, LF was detected by immunocytochemistry in the cell nucleoli already after 30 min of incubation with the THP-1 cells and found by electromobility shift assay to decrease the binding of nuclear factor (NF)-κB to the TNF-α promoter. The antimicrobial activity against *E.coli*, *S.aureus* and *C.albicans* of synthetic peptides homologous to the surface exposed α-helix and β-sheet region from the N-terminal end of human LF showed that a short region comprising 12-15 a.a. corresponding to the major part of the helix region were optimal for the killing activity against all three microorganisms. In addition certain amino acids such as cystein, and hydrophobic and positively charged amino acids in the 12-amino acid long peptide were found to be important for the expression of antimicrobial activity.

In summary, orally given LF can reduce infection and inflammation in a remote site such as the urinary tract, and mediates anti-inflammatory activities in the colon. This dual effect may partly reside in the antimicrobial region of the LF molecule, since synthetic fragments also provide similar activities. One possibly important mechanism of its anti-inflammatory effects is through the ability to down-regulate cytokine production via interference with the transcription factor NF-κB. The anti-infectious and anti-inflammatory activities of LF on mucosal surfaces is being utilized by the suckling child which obtains large amounts of LF via the maternal milk. However, therapeutic use of LF or its fragments may be possible in other fields of application.

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Lactoferrin and lactoferricin octopus cover illustration: Agneta Rooth

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Original papers

This thesis is based on the following papers, which will be referred to in the text by their roman numbers:

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In manuscript

To my mother

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ABBREVIATIONS

a.a.	amino acid
apoLF	apolactoferrin
Arg	arginine
bLF	bovine lactoferrin
bLFcin	bovine lactoferricin
BSA	bovine serum albumin
CD	cluster of differentiation
DX	dextran-sulphate
hLF	human lactoferrin
hLFcin	human lactoferricin
holoLf	iron saturated (holo) lactoferrin
HSA	human serum albumin
IL	interleukin
INF-γ	interferon gamma
LBP	lipopolysaccharide binding protein
LF	lactoferrin
LFcin	lactoferricin
LPS	lipopolysaccharide
mLF	mouse lactoferrin
NF-κB	nuclear transcription factor kappa B
PBS	phosphate buffer saline
PBS-T	phosphate buffer saline containing 0.05% Tween 20
PMN	polymorphonuclear leucocytes
UTI	urinary tract infection
TLR4	Toll like receptor 4

INTRODUCTION

Lactoferrin

Lactoferrin (LF), a metal binding glycoprotein present in milk, mucosal secretions and secondary granules of neutrophils is an innate defense factor having antimicrobial and immunomodulatory activities. LF exerts broad-spectrum antimicrobial activity against bacteria, fungi and viruses (1, 2). The regulation of immune responses by LF is a result of its ability to interact and affect the functions of the immune cells (3). Due to its high content in exocrine secretions and human milk, especially in colostrum, where it makes up 43% of the total protein, LF may constitute an important factor in the mucosal host defense and particularly in the infant.

Distribution of LF

LF is present in the milk of all mammalian species (4). The levels in milk depend on the lactation period, being more elevated in colostrum than in transitional and mature milk (Table 1) (5-7). In man the milk contains high concentration of LF, several order of magnitude higher than in cow's milk (4-6, 8). A human infant receives approximately 1-7 g of human lactoferrin (hLF) per day. The LF level in milk of other species varies (Table 1) (4).

External secretions of the respiratory, gastrointestinal and genitourinary tracts contain LF (Table 1). The levels of hLF are high in tears and genitourinary tract secretions, where they make up e.g. 20 and 10% of the total protein in the vaginal mucus and seminal plasma. Moderate levels are found in the respiratory and gastrointestinal tracts, and synovial fluid (1).

The secondary granules of neutrophils also contain LF, which is released during inflammatory conditions (9). Cytokines (IL-1β, TNF-α) (10, 11), LPS, (12), and immune complexes (13) induce the release of LF from neutrophils. The plasma hLF level which is normally very low, can increase several orders of magnitude in infections and tumors reaching a concentration of e.g. 0.2 mg/ml in acute sepsis and 14 mg/ml in infected parotid fluid (12). It is likely that at sites of inflammations its concentration may reach milligrams per milliliter (14).

LF gene and synthesis

The hLF gene is located on chromosome 3q21 (15-17), the bLF gene on chromosome 22 (18), and the mouse LF gene on chromosome 9 (15). The LF gene is organized in 17 exons and the size varies depending on species between 23 and 35 kb (19-24). The gene encodes for 711 and 708 a.a. in hLF and bLF, respectively. The first 19 a.a. residues of both proteins code for the signal peptide, the rest constituting the mature protein. The expression of the LF gene is cell-, tissue-, embryonic stage-, and hormone dependent as well as species-specific (25). Mutations and lower expression of the LF gene during oncogenesis compared with normal conditions has been observed (20, 26). A high mRNA expression of LF is found in normal human mammary gland, stomach and genitourinary tract (kidney, vagina, uterus, prostate, testis).

Table 1. Distribution and levels of LF

Distribution			mg/ml
Milk	human	*colostrum*	5-7
		transitional	3.7
		mature milk	1-3
	rat, rabbit,dog		<0.05
	cow, goat, sow		0.001-0.1
	mouse, guinea pig, mare		0.1-1
Human secretions		*tears*	0.4-2.2
	respiratory tract	*nasal secretion*	0.1
		saliva	0.005-0.01
		bronchial mucus	0.03-0.04
	gastrointestinal tract	*duodenal juice*	0.00007-0.000172
		pancreatic juice	0.000032-0.000072
		bile	"
		small intestine	"
	genitourinary tract	*vaginal mucus*	0.004-0.2 per mg protein
		uterine secretions	0.5-1
		seminal plasma	0.4-2
		urine	0.001
		amniotic fluid	0.002-0.032
Synovial fluid			0.01-0.08
Neutrophils			0.002-0.006 per 10^6 neutrophils
Blood			0.00009-0.0015

In contrast, no expression of LF is noted in the mouse kidney or stomach, while a higher expression is observed in the murine, than in human lung (25). In the endometrium and

vagina the expression is up-regulated by estrogen (27), while in the mammary gland by prolactin (28).

LF is synthesized by epithelial cells of the mammary (29), lacrimary (30) and salivary glands (31), biliary tract (32), pancreas (31), uterus, cervix, vagina and prostate (27). LF present in neutrophils is synthetized during the transition of cells from promyelocyte to myelocyte (33). In mice, the protein was detected in the gestation in neutrophils of the fetal liver (embryonic day 11) and in the epithelium of the digestive and respiratory systems (embryonic day 16 onward) (34).

Both hLF and bLF gene polymorphism exist (20, 35). hLF gene polymorphism has recently been reported within the exons 2, 5, 7, 9, 13, 14 and 15 of normal individuals (26, 36). The polymorphism in the exon 2, that affects the a.a. residues 11 (A or T) and 29 (K or R) of hLF is of importance with respect to the antimicrobial activity of the protein against *S. aureus* and *Candida* (36). Thus, the *in vitro* antibacterial activity of hLF from individuals homozygous for AK is ten times higher than of those homozygous for TR (36).

Isoforms of hLF, bLF and mLF are reported (37-41). Their existence is possibly a consequence of LF gene polymorphism and differential glycosylation of LF. Three hLF isoforms, hLF-β and hLF–γ with RNAse activity and iron binding property, and hLF–α with iron binding, but no RNAse activity have been isolated and characterized from milk and secondary granules of neutrophils (37, 38). Also, a delta hLF mRNA, a product of alternative splicing of the hLF gene, was detected in normal tissues having the highest expression in spleen, pancreas, colon, kidney and lung (42). The biological significance of the isoforms is presently unknown.

Structure of LF

LF belongs to the transferrin family of proteins having the structure closely related to transferrin. The a. a. sequence of different LF species was resolved by sequencing and cDNA cloning, while the three dimensional structure was resolved by X-ray crystallography (43-48). LF is constituted of a single polypeptide chain that comprises two homologous lobes, the N and C lobe. Each lobe contains one metal binding site and consists of two domains (N1 and N2, C1 and C2, respectively), which are linked by an α helix region. The domains form a cleft where 2 ferric ions (Fe^{+3}) can bind synergistically with a bicarbonate anion (CO_3^{2-}). The iron-saturated form is called hololactoferrin (holoLF), while the iron-depleted form is called apolactoferrin (apoLF). There is high homology in the a. a. sequence of LF from different species. Thus, hLF shows 69% homology with bLF and 70% with mouse LF, respectively (46, 49). hLF contains 691 a.a. according to one report (47), or 692 a.a. according to another one (43). It has a molecular weight of 78 kDa and two glycosylated sites (Asn 138 and Asn 490) containing N-acetyl and poly N-acetyl lactosaminic type of glycans with structural microheterogeneity (50). The absence of β1-3 and 1-6 linked fucose residues and the homogeneity of the glycans in the neutrophil hLF constitute the difference with milk hLF. The N and C-globular lobes contain the amino acids 1-332 and 345-692 respectively. These are linked together by the α helix comprising the a.a. 333-344 (47). The protein moiety, especially the N terminal domain of the molecule mediates most of the biological activities of hLF (1). The two iron binding sites of hLF are similar. Iron binds to a.a. located in the

positions Asp 60, Tyr 92, Tyr 192 and His 253 in the N lobe, and Tyr 435, Tyr 528, His 597 and Asp 395 in the C lobe, and two oxygens from the anion CO_3^{2-}. It is estimated that only 6-8% of hLF in milk is iron saturated. The hLF is secreted into the exocrine fluids or released from the secondary granules of neutrophils as apoLF (51). The three dimensional structure of hLF is shown in Fig 1. bLF contains 689 amino acids (46) and 4 glycosylated sites with N acetyllactosaminic (Asn 368, Asn 476) and oligomannosidic type of glycans (Asn 233 and Asn 545 Asn 368, Asn 476). The iron binding sites of bLF are located in the same positions and identical a.a. as in the N lobe of hLF, and Asp 395, Tyr 433, Tyr 526 and His 595 in the C lobe (48, 52). bLF has an extra disulphide bridge (160-183), not present in hLF (53). Mouse LF consists of 688 a.a. and has only one N-acetyllactosaminic type of glycan (49, 54).
LF binds iron with a higher affinity than transferrin and retains it at lower pH (55). In the absence of iron, LF is able to bind other metals like copper, manganese, zinc, aluminium and gallium (56-60).

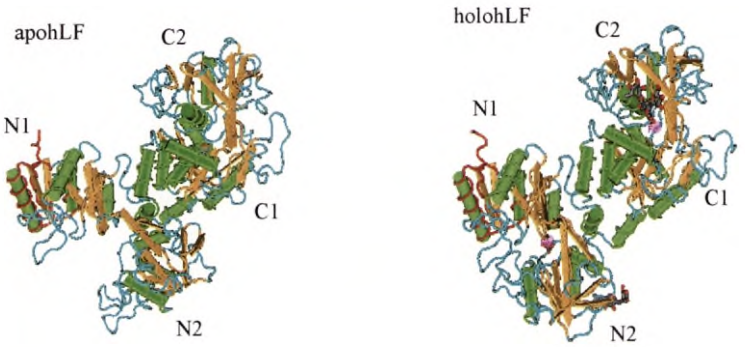

Fig. 1. The three dimensional structure of apo-hLF and holo-hLF. The ferric ions in holo-hLF are indicated by pink spheres. The glycosylation sites are only shown in holo-hLF. The region corresponding to the hLFcin fragment is highlighted in red.

LF metabolism

LF in milk is passing through the gastrointestinal tract of the suckling newborn. Little is known about the extent of LF degradation and the digestive segment responsible for it. LF and fragments were found in stools and urine of breast and formula fed human infants, suggesting the absorption of partially degraded protein in the gut (61-66). Receptors for LF are present in the small intestine of human infants (MW110 kDa) and adults (67), rhesus monkeys (68), mice (MW 130 kDa) (69, 70), rabbits (MW 100 kDa) (71), and piglets (72, 73). While the human receptor is specific for hLF not binding bLF, the mouse receptor binds mLF, hLF, and bLF with similar affinities (70, 74). The biological role of the receptor is not elucidated, although there it is some evidence that is involved in the regulation of iron uptake in the gut (75). *In vitro* studies have shown that LF is relatively resistant to proteolysis by

trypsin and chemotrypsin and the iron saturated LF is more resistant than the apo form (76). However, a pepsin derived fragment, called lactoferricin (LFcin) was generated from LF *in vitro* (77). bLFcin was found in the gastric content of an adult male as early as 10 min after oral feeding of bLF (78). The amount of LF surviving digestion in the gastrointestinal tract as intact protein was observed to decrease with age as observed in children (75). It still remains an open question to which extent the LFcin would be generated in the gastrointestinal tract of milk or LF-fed newborns, since the enzymatic digestive capacity of the infant is lower than in adults (low concentration of pepsin and other pancreatic enzymes, a higher gastric pH) (75).
bLF and bLF fragments were also found in the gastrointestinal tract of animals fed bovine milk or milk supplemented with bLF. Thus, partly degraded bLF was detected in the stomach and small intestine of adult rats, and bLF fragments containing bLFcin were found in the faeces of adult mice fed milk enriched with bLF (79, 80).
LF released from neutrophils is transported to the liver where it is taken up by hepatocytes through asialoglycoprotein receptor mediated endocytosis and then released as degraded forms (81, 82). By binding with low affinity to chondroitin sulphate and specifically via residues 25-31 to the lipoprotein receptor related protein (LRP), LF can also be cleared from the circulation by the liver parenchymal cells (83, 84).

Antimicrobial effects of LF

Antibacterial effects of LF

Several studies have shown bacteriostatic and some bactericidal effects of LF against Gram negative and Gram-positive bacteria *in vitro*. The binding of LF to bacteria is suggested to be a prerequisite for its antibacterial effect (85). LF has shown to bind to *E.coli, Prevotella intermedia, P. melaninogenica,* and *Porphyromonas gingivalis,* and *Streptococcus pyogenes* (86-89). Specific receptors are reported for LF on *S.aureus* (MW 450 kDa), *Neisseria* and *Moraxella* species (MW 100 kDa), as well as *Haemophilus influenzae* (105 kDa) (87, 90-92).
LF can exert its antibacterial effects by different mechanisms. One of the first described was withdrawal of iron, an essential bacterial nutrient, the antibacterial activity being restricted to the apoLF with holoLF inactive. However, some Gram negative bacteria can overcome the iron withholding effect of LF by synthesizing siderophores, which remove iron from LF (93). Also, some pathogenic bacteria e.g. *Neisseria* species utilize the iron from LF by expressing receptors for LF that can internalize the iron saturated form of the protein (91, 92, 94). These receptors are species specific, thus human pathogens can only use hLF as source of iron (95).
LF acts synergistically with lysozyme and sIgA against bacteria (96-99).
LF exerts the antibacterial activity by interacting with the bacterial cell envelope structures of both Gram-negative and Gram-positive bacteria. The cell wall of Gram negative bacteria is composed of an asymmetric outer membrane and a periplasm (100) (Fig. 2 A). The outer leaflet of the outer membrane is composed of lipopolysaccharide molecules (LPS) (approximately $3.5X10^6$ per cell in *E.coli*), lipoproteins and proteins and the inner leaflet of mainly phospholipids. LPS is an amphiphilic molecule composed of the O–specific chain consisting of repeating oligosaccharide units; the core region containing the sugar 2-keto-3 deoxyoctulonic acid, heptose, and free phosphate groups; and lipid A, a glycolipid inserted into the membrane, composed of a biphosphorylated β (1-6) linked D-glucosamine

disaccharide and up to seven long chain fatty acids. There are, however, structural variations in the lipid A part. Divalent cations (Ca^{2+} and Mg^{2+}) are also integrated in the outer leaflet of the membrane in order to stabilize the anionic character of the core oligosaccharide. The outer membrane contains the outer membrane proteins (OMPs), including porins (e.g. OmpC, OmpF and PhoE of *E.coli*), which form pores accounting for the permeability to hydrophilic molecules (101, 102). The periplasm is constituted of a thin, rigid layer of peptidoglycan (or murein), a lipoprotein attaching the peptidoglycan to the outer membrane, and a variety of hydrolytic enzymes. Essential for maintenance of the three dimensional shape of bacteria, the peptidoglycan is made up of a polysaccharide (polymer of N-acetyl-glucosamine and N-acetyl-muramic acid) cross-linked by peptides. The inner membrane has a phospholipid bilayer structure, which unlike eukaryotic membranes, does not contain cholesterol. The inner membrane contains integrated membrane transport proteins, ion pumps and enzymes.

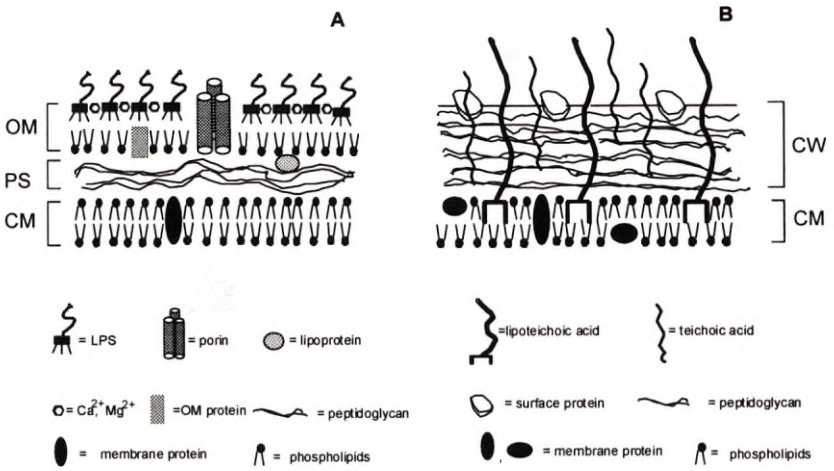

Fig. 2. Schematic representation of the cell envelope in Gram-negative (A) and Gram-positive bacteria (B). OM, outer membrane; PS, periplasmic space; CW, cell wall; CM, cytoplasmic membrane.

In Gram negative bacteria, both hLF and bLF bind to and release LPS, destabilizing the outer membrane and increasing the bacterial killing by lysozyme (103, 104). Several studies have shown that LF binds to LPS and to the lipid A moiety, and the a.a. residues 1-5 and 28-34 of hLF are involved in this binding (105-109). LF binds via a.a. residues 1-5, 28-34, and 39-42 to the *E.coli* porins (e.g. OmpF, OmpC and PhoE), thus affecting the bacterial outer membrane permeability (110 , 111). It is believed that LF destabilizes the outer membrane of Gram negative bacteria without penetrating it (103, 112).

Unlike Gram negative bacteria, Gram positive bacteria do not have an outer membrane as a part of their cells wall and being multilayered, their peptidoglycan is much thicker (113) (Fig 2 B). As a part of the Gram positive cell wall, the teichoic and lipoteichoic acid polysaccharides are linked by phosphodiester bridges to the N-acetyl muramic acid of the

peptidoglycan and covalently linked to the glycolipid of the cytoplasmic membrane, respectively (Fig. 2B).

In Gram positive bacteria hLF interacts with lipoteichoic and teichoic acid (114). The binding, neutralization of negative charges, and release of lipoteichoic acid from *Staphyloccocus epidermidis* by hLF, with a subsequent increase in the susceptibility to lysozyme was shown (114). hLF seems to induce intracellular changes in bacteria (e.g. affecting nonspecific esterase activity) without affecting the cytoplasmic membrane permeability (115). The first two arginine residues in position 2-3 (Arg^2-Arg^3) are important in the killing activity of hLF against *S. aureus* bacteria (115).

A third mechanism of antimicrobial activity of LF is the release of the microbicidal LFcin fragments upon enzymatic hydrolyzis (116). They are more potent in killing Gram negative and Gram positive bacteria than the native proteins. The structure of LFcin is shown in Figs. 1 and 3.

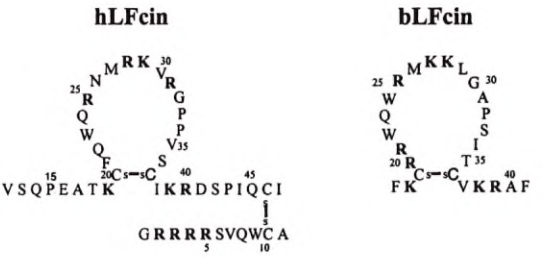

Fig 3. Amino acid structure of hLFcin and bLFcin. Single letter code is used to indicate the amino acid sequence. Basic amino acids are indicated in bold.

On a molar basis, hLFcin and bLFcin is 2-and 12-fold more active than the parent molecules and bLFcin 9-fold more effective than hLFcin against *E. coli* O111 (77). hLFcin corresponds to the a.a. residues 1-47 of the N1 terminal domain of hLF. This fragment contains the antibacterial (a.a. residues 18-40) and the LPS binding domains (residues 1-5 and 28-34) (77, 107). bLFcin corresponds to a.a. residues 17 - 41 with a disulphide bond between a.a. 19 and 36. The a.a. residues 17-28 adopt an α helix, 29-31 a turn, and 31-41 a β sheet in the native protein (77). However, by NMR, bLFcin has been shown to adopt a distorted antiparallel β-sheet (117). In aqueous solutions the peptides adopt random conformations. The loop region of hLFcin (a.a. 20-37) resides in an exposed surface of the N1 domain of hLF and adopts an α helix (a.a. 21-31) with the hydrophobic tail (a.a. 32-36) in the native protein (118). The synthetic peptide corresponding to a.a. 21-31 of hLFcin was found more active than the entire loop region, while the peptide corresponding to the hydrophobic tail was found inactive against several bacteria (118). The peptide 21-31 showed an increased bacteriostatic effect against *Staphylococcus aureus* compared to *E.coli* (118). The a.a. residues 1-17 of hLFcin were initially shown not to be important in the antibacterial activity. However, a recent study has shown that the synthetic peptide containing the first cationic domain of hLFcin (a.a. 1-11) is even more active than the peptide containing the second cationic domain (21-31) (77, 115). The peptide 1-11 has even found more potent *in vivo* than *in vitro,* when given intravenously

to mice 24 hrs after an experimental *S. aureus* or *K. pneumoniae* infection induced in muscle (115).

The bactericidal mechanism of LFcin and synthetic peptides is not well defined. The synthetic peptide corresponding to a.a. residues 21-31 of hLFcin binds to LPS, enters to and disrupts the outer membrane of *E.coli* O111 (118). The synthetic peptide corresponding to residues 18-40 of hLFcin interacts with the inner membrane of *E.coli* O111 (119). The peptide 1-11 is suggested to affect the membrane permeability of *S. aureus* (115).

As the native protein, bLFcin destabilizes the outer membrane of Gram negative bacteria and releases even more LPS from the membrane than bLF (112). In Gram positive bacteria bLFcin binds to teichoic acids (120). bLFcin also acts on and affects the permeability of the cytoplasmic membranes of Gram positive and Gram negative bacteria (116, 121). bLFcin has recently been shown to cross the cytoplasmic membrane and reach the cytoplasm of *E.coli* and *S. aureus* (122).

Through its oligomannosidic type of glycans, bLF inhibits the adherence of type 1 fimbriated *E.coli*, and *Helicobacter* bacteria to the host cells (123, 124).

The antibacterial role of LF, especially of hLF or peptides is less studied *in vivo*. LF protects mice against systemic experimental *E.coli* and *S. aureus* infections (125, 126). Thus, bLF given intravenously 24 hrs before the systemic *E.coli* challenge reduced the lethality of the animals and the number of bacteria in the kidneys and lung (125). hLF given intravenously (i.v) and bLF given i.v. or orally one day before intravenous inoculation with *S. aureus* significantly reduced the number of bacteria present in kidneys after 14 days of infection (126). Daily oral administration of bLF for 4 weeks starting three weeks after oral inoculation with *Helicobacter pylori* decreased the number of bacteria in the stomach of Balb/c mice (124). The effect was attributed to the inhibitory activity of bLF on the adherence of bacteria to the gastric epithelium (124). In an experimental model of colonization with *Clostridium*, a decreased number of bacteria was found to translocate to the mesenteric lymph nodes and to be present in the feaces of mice fed milk supplemented with bLF or pepsin-derived bLF hydolysate, indicating bacteriostatic effect of bLF in the gut (123, 127).

A protective effect of orally given LF against enteric infections in neutropenic human individuals was reported (128).

Antifungal effects of LF

Fungistatic and fungicidal effects mostly against *Candida albicans* have been described for hLF, bLF, bLFcin, a peptide based on bLFcin (a.a.17-26), and a peptide based on hLFcin (a.a. 1-11) (129-133). The apoLF is more effective than holoLF, and the LFcins more than the native proteins with respect to the antifungal activity (129, 130 , 133).

The outer cell wall layer of *Candida albicans* is composed by a phosphomannoprotein complex constituted by mannan (polymers of mannose) covalently linked to proteins and containing 1-2% phosphate. The structural components of the cell wall are β-glucans (branched polymers of β1-3 and β1-6 glucose) as main constituents and chitin (unbranched polymer of β1-4 bound N-acetyl-D glucosamine) as minor constituent. In addition, the cell wall contains proteins and lipids (134). Unlike bacteria, the cytoplasmic membrane of fungi

contains ergosterol. The schematic composition of *C. albicans* cell wall and cytoplasmic membrane is shown in Fig. 4.

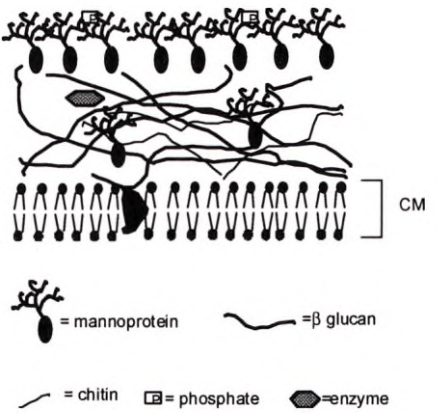

Fig. 4. Schematic representation of C. albicans cell wall and cytoplasmic membrane. CM, cytoplasmic membrane.

hLF interacts with the mannoproteins from the cell wall of *Candida albicans* (135) and induces fungal cell surface alterations, formation of surface blebs and leakage of proteins (130). A hLF peptide corresponding to the first 11 a.a. of hLF (1-11) was recently found more effective in killing *C. albicans* than a peptide corresponding to a.a. 21-31 of hLF (133). The a. a. residues Arg^2-Arg^3 of the peptide 1-11 and the peptide-induced extracellular release of fungal ATP are important in the killing activity (133). The optimal binding of bLFcin to *Candida albicans* at pH 6 and in the absence of Ca^{2+} and Mg^{2+} correlates to its killing capacity (131). Synergistic effects of hLF, bLF and bLFcin with antifungal drugs e.g. clotrimazole and fluconazole has been reported (136, 137).

Immunomodulatory and anti-inflammatory effects of LF

Inflammation

Inflammation is a complex process by which a tissue responds to a damage or an infection. An increase in the blood supply and capillary permeability, and the migration of leukocytes and serum proteins to the area occurs initially. In general, the neutrophils are the first cells arriving at the site of an acute inflammation followed by monocytes and activated lymphocytes. $CD8^+$ T cells and B cells usually arrive later. The chemokines, such as IL-8 synthetised by the cells present in the tissue or by endothelial cells trigger the migration of neutrophils to the inflammatory site. Mononuclear phagocytes release proinflammatory cytokines such as TNF–α, IL-1β and IL-6. IL-1β and TNF-α induce the expression of E selectin, and ICAM–1 and VCAM-1 adhesion molecules on the endothelium. ICAM-1 and VCAM-1 molecules bind to LFA-1 ($\alpha_L\beta2$), VLA-4 (α4β1) and CR3 (α M β2) integrins on

leukocytes, resulting in the cell migration through the endothelium to the tissue. IL-1β and TNF-α also induce the production of IL-8 by macrophages and endothelial cells. The cells arrived at the site of inflammation release mediators that activate and recruit other cells. Activated macrophages produce MIP-1α and leukotriene LTB4 which being chemotactic, attract more monocytes to the tissue. The lymphocytes release INF-γ and TNF-β which activate macrophages and enhance their phagocytic activity.

The complement, the clotting, the fibrinolytic, and the kinin systems are serum plasma molecules involved in the inflammatory response. Auxiliary cells like platelets, mast cells and basophils are sources of mediators such as histamine and serotonin, which are important in vasodilation and vascular permeability. An acute local inflammation is accompanied by a systemic response, IL-1β, TNF-α, IL-6 inducing the production of the acute phase proteins by the liver. If the stimuli or the infection is not cleared, an acute inflammatory response can develop into a chronic one (138).

LPS as a potent inducer of cytokines in monocytes

Monocytes/macrophages are important cells of the innate immune system that respond to bacteria and bacterial components by secreting cytokines, mediators like NO, and reactive oxygen species. LPS, the main component of the outer membrane of the Gram-negative bacteria stimulates monocytes/macrophages to produce cytokines like IL-1, TNF–α, IL-6, IL-10 and chemokines like IL-8. LPS binds to LBP present in serum at 3-10 μg/ml, which then, catalyze the transfer of LPS to the CD14 receptor (139). CD14 receptor does not posses a transmembrane domain, being expressed on the surface of the myeloid cells via a glycosylphosphatidylinositol tail (membrane CD14) which anchor the protein in the membrane (140). CD14 is also present free in plasma (2-6 μg/ml) as soluble CD14 (sCD14) and mediates LPS activation (141). LPS then interacts with the signaling receptor Toll like receptor 4 (TLR4) and the accessory protein MD-2 (142). As a result of this interaction the NF-κB and three MAPK kinases (p38, JNK, ERK1/2) signal pathways are activated (142). NF-κB is a transcription factor involved in the transcription of many proinflammatory cytokine genes (IL-1β, TNF-α, IL-6, IL-8, IL-12). NF-κB exist in the cell cytoplasm in an active form consisting of the heterodimers p50/p65 associated with the inhibitory unit IκB (143). Upon LPS stimulation, the phosphorylation of IκB occurs, NF-κB nuclear localization signal is revealed, the transcription factor translocates to the nucleus, binds to and activates transcription of the target cytokine genes (144).

The effect of LF on immune cells

LF contributes to the host defense mechanisms not only through its anti-microbial effects, but also by modulating the immune system via its ability to bind to and affect the functions of immunocompetent cells. LF has effects on the majority of the cells of the immune system (monocytes/macrophages, Langerhans, neutrophils, γδ T cells, NK cells, platelets, B and T cells) (1, 145-147). The underlying mechanism of most of its activities is unknown. Some of the effects are dependent, while others independent on the iron saturation status of LF (1).

Being a cationic protein, LF binds to the cells in two ways. The charge-charge interaction of LF with proteoglycans, or membrane DNA accounts for a low affinity binding of the protein

to the cells. A higher affinity, but lower capacity of binding to the membrane receptor(s) constitutes the second way of interaction of LF with cells.

LF effects on monocytes

Due to its high concentration at mucosal surfaces and release from neutrophils at sites of inflammation, it is very likely that LF interacts with mononuclear phagocytic cells *in vivo*. Low and high affinity binding sites for LF are present on human blood monocytes (148-150), monocytic cell lines e.g. THP-1 (151-153), U937 (154), monocyte/macrophage differentiated HL-60 (105), as well as human alveolar (155) and mouse peritoneal macrophages (156). The binding is independent on the iron content of LF (151, 157). In all studies milk, not neutrophil derived LF is used. This could be of relevance for interpretation of binding to macrophages of hLF released from neutrophils, since the fucose residue present on milk, but not on neutrophil hLF could be involved in binding to mannose receptors present on macrophages. The binding of LF to the monocyte proteoglycans, present in a higher amount on these cells than on other cell types, could explain the ionic and low affinity interaction of LF with monocytes. An ionic interaction in binding of hLF to alveolar macrophages is described (155). The basic cluster of four arginine residues in position 2-5 (Arg^2-Arg^5) and the a.a. residues 28-31 of hLF is involved in the binding to glycosaminoglycans (158). Several hLF-binding proteins on THP-1 cells (MW of 35, 50 and 80 kDa) exist (152). hLF and bLF bind with similar affinity to THP-1 cells. The binding is mediated mainly by the protein moiety of hLF, and to a minor extent via its polylactosaminoglycans (152, 153). The cytokine secretion and the phagocytic capacity of monocytes/macrophages are affected by LF in experiments *in vitro*. Iron saturated LF has been proposed to inhibit myelopoiesis through down regulation of GM-CSF via inhibition of monocytic IL-1β (159-162). LPS-induced IL-1β, TNF-α, and IL-6 in human and murine monocytes and monocytic cell lines are down regulated by LF. IL-6 is also inhibited by bLFcin as shown *in vitro* (145, 163, 164). Binding of LF to LPS and soluble CD14, as well as competition with LBP for binding to LPS could partly explain the inhibitory activity of LF on cytokine secretion (105, 109, 165). LF also inhibits the PMA-induced prostaglandin E2 production of human breast milk macrophages (166). Although the internalization of hLF in monocytes/macrophages is shown by several studies (150, 167-170), the fate of the protein intracellularly is not fully elucidated. Studies using subcellular fractionation techniques showed the internalization of iron saturated hLF into a myeloperoxidase-positive lysosomal fraction (170), and of apoLF in the cytosolic fraction (150). By immunoelectron microscopy iron saturated hLF was detected in the endoplasmic reticulum and in the nucleus (169). After binding and uptake of iron saturated hLF into monocytes, 50% of its iron is transferred to the cytosolic ferritin. Thus hLF was proposed to participate to the hyposideraemia of infections (167, 170, 171). However, this debated effect (154) seems less likely to occur *in vivo*, since the ability of LF to remove iron from plasma transferrin and deliver it to the macrophage ferritin is extremely slow (172). Moreover, the IL-1β-induced hyposideraemia can be seen in neutropenic mice, indicating that neutrophil derived LF does not contribute to this condition (173).

Increased uptake and killing of intracellular *Trypanosoma cruzi* and *Listeria monocytogenes* parasites by murine macrophages and human monocytes in the presence of hLF were

reported. The intracellular killing, but not the uptake of the microorganism was dependent on the iron saturation of hLF (174, 175). Iron delivery by LF to an oxygen radical generating system in an acidic environment such as a phagolysosome seems to be responsible for the mechanism of killing (1, 175). However, at normal extracellular pH, apoLF acts rather as an iron scavenger and inhibits the radical production, thus protecting the cells from oxidative damage (1, 150).

LF effects on neutrophils

Neutrophils are the first cells recruited to the site of infection and inflammation. LF affects *in vitro* important functions of these cells like mobility, hydrogen production and killing of some microbes. Thus, LF possibly affects the migration of the cells to the inflammatory sites *in vivo*. As for monocytes/macrophages, low and high affinity binding sites of milk hLF on human blood neutrophils are reported (176, 177). However, the binding of hLF to neutrophils is approximately ten times lower than to macrophages or lymphocytes (177). By binding to neutrophils, hLF can reduce the charge of the cells and promotes their adherence to the tissue (178). hLF increases the mobility and superoxide production induced by stimuli such as zymosan, PMA and FMLP in human neutrophils, althought the killing of *S.aureus* is not affected (179). While the mobility of the cells is increased regardless of the iron saturation of LF, the generation of superoxide by iron saturated LF is higher than for native LF (179). The phagocytic activity of human blood neutrophils is stimulated by bLF and bLFcin as determined by incorporation of latex beads (180). An augmentation of neutrophil killing capacity of *Candida albicans* in the presence of bLF, bLfcin and a bLFcin-derived peptide was also shown (132, 181). By its ability to bind to LPS, hLF prevents the binding of LPS to L-selectin and the production of reactive oxygen species from neutrophils (165). hLF protects the neutrophils from oxidative damage by inhibition of lipid peroxidation (182) possibly by its ability to bind iron and inhibit hydroxyl radical production at physiologic pH (1).

LF effects on T cells

LF affects the proliferation, differentiation and cytokine production of T cells. LF was shown both to inhibit (183, 184) and stimulate the proliferation of T cells (185-188). This dual effect seems to be modulated by the environmental conditions, LF affecting the T cell proliferation by regulating the iron uptake, the effects being inhibitory at low iron levels and stimulatory in excess of iron (189, 190). LF has also been shown to induce the differentiation and maturation of T cells (191, 192).

The effects of LF in T cells are possibly mediated via the receptor described on the cells. The only hLF receptor described on T cells is a glycoprotein with MW of 105 kDa (186, 193). The receptor recognizes the residues 4-52 in the N1 terminal domain of the hLF, which forms two exposed loops with a βαβα structure (147).

LF effects on B cells

LF affects the proliferation and differentiation of B cells. Both hLF and bLF have shown to increase proliferation of human, but not mouse cell lines in serum free medium (185). Iron saturated hLF induces the maturation of spleen B cells isolated from 7-8 days newborn mice as detected by increased expression of IgD and complement receptors (C_3R) maturation

markers on these cells (194). Iron saturated hLF also enables the spleenic B cells from normal Balb/c newborn and adult CBA/N deficient mice (lacking B cell function as antigen presenting cell) to acquire *in vitro* the antigen presenting cell function (194). LF and bLFcin have recently shown to bind to CpG bacterial oligonuleotide and to inhibit the binding and internalization of these immunostimulatory molecules in B cells (195).
The a.a. Arg^2-Arg^3 residues at the N terminal domain of hLF are important in the interaction of LF with B cells (196).

LF effects on NK cells
The augmentation by LF of NK cells cytotoxicity (146, 197, 198), inhibition of the tumor growth (199), and of angiogenesis (200), may partly explain the reported antitumoral activities of LF on colon, urinary bladder, esophagus and lung carcinoma in animal models (201-205).

LF effects on platelets
The inhibition of platelet aggregation and thus an anti-coagulant effect of LF was reported by *in vitro* studies (147). This inhibition could be due to the structural analogy between the sequence target of the platelets GpIIb-IIIa complex (RGDS), and the residues 39-42 (KRDS) of hLF (206). The LF receptor on the surface of platelets is similar to the lymphocytic receptor (147).

Anti-inflammatory effects of LF in vivo
There are few reports studying the anti-inflammatory effects of LF *in vivo*. The most explored *in vivo* model is endotoxemia. Thus, intravenous administration of bLF to mice not earlier than 24 hrs before the challenge with LPS decreased the TNF–α levels in serum (207). Protection against lethal endotoxic shock in germfree, colostrum-deprived piglets using repeated oral administration of bLF before intravenous LPS challenge was reported (208). The ability of bLF to block the LPS *in vivo* was suggested to be a consequence of inhibition by bLF of LPS binding to piglet monocytes, as shown by *in vitro* studies (208). hLF administered intraperitoneally 1 h before LPS challenge reduced the mortality in mice and protected the small intestine from the damages induced by LPS (209). Topical application of hLF on skin inhibited allergen-induced cutaneous inflammation in mice and human volunteers (210-212). Thus, oxazolone- and diphenylcyclopropenone-induced Langerhans cell migration from epidermis and dendritic cell accumulation in the draining lymph nodes were inhibited by topical application of hLF via down-regulation of TNF–α produced by keratinocytes (210-212).

LF and transcription
Using the K565 cells and human blood monocytes hLF was shown to be taken up and transported to the nucleus (169, 213). Recently, a peptide corresponding to the a.a. 1-5 of hLF was found to be localized to the nucleoli of various cell lines such as cervix epithelial, (HeLa), glioblastoma (U87MG) and bladder carcinoma (5637) cell lines (214). This peptide was proposed to be the nuclear localization signal of hLF (214).

hLF binds to three specific DNA consensus sequences and regulates the transcription of the reporter genes containing these sequences in the promoter region (215).

Urinary tract infection (UTI) mouse model

The urinary tract is normally sterile. Several factors such as urine flow, bactericidal and anti-adherence molecules present in the urine (like lysozyme, LF, defensins, IgA, uromodulin, low molecular oligosaccharides) contribute to maintain this sterility (216). However, uropathogens have developed strategies to overcome the defense mechanisms, by adhering and colonizing the urinary tract epithelium. They induce a local inflammatory response in susceptible individuals. The large intestine, vaginal introitous, and periurethral area are the sources of the *E. coli,* which is the most common uropathogen (217). After the ascendance up into the urinary tract, bacteria may cause different manifestations of UTI that vary in pathogenesis and severity, such as asymptomatic bacteriuria, acute cystitis or acute pyelonephritis (217).

The interplay between the bacterial virulence factors and the host response to infection reflected by the severity of the UTI was studied in detail using a mouse model (218-221). The adherence of bacteria via fimbriae to the urinary tract epithelium constitutes the first step in the pathogenesis of UTI (222, 223). The uroepithelial cell receptors for type P fimbriae consist of glycoshingolipids of the globoseries (Galα1-4βGal containing oligosaccharides bound to ceramide in the cytoplasmic membrane), while type 1 fimbriae binds to mannosylated glycoproteins (224, 225). Using TLR-4 proficient (C3H/HeN) and defective (C3H/HeJ) mice and different mutated bacterial strains, LPS, bacterial fimbriae, especially type P, but also type 1, have shown to be important for inducing a local mucosal inflammatory response characterized by the secretion of the pro-inflammatory cytokines IL-6 and IL-8, and the subsequent recruitment of PMNs into the urine (220, 223, 226, 227). Type P and 1 fimbriated bacteria utilize different signaling pathways for activating the uroepithelial cells, that have recently been elucidated (219, 220, 227). P fimbriated bacteria induce a cytokine response in epithelial cells by utilizing the ceramide signal pathway, and TLR4 as coreceptor (219, 227), while type 1 fimbriated bacteria trigger one LPS and TLR-4 dependent, and one lectin-dependent but TLR-4 independent signaling pathway (220). The innate immunity provided the most efficient defense mechanism against UTI, since by depleting the neutrophils the host resistance to infection decreased (228). Thus, C3H/HeJ mice responded to bacterial infection with much lower number of influxing neutrophils and IL-6 levels in the urine and, unlike the normal mice, that normally clear the infection within 3-7 days, they remained infected (229). IL-8 and IL8 receptor are involved in the recruitment and migration of neutrophils across the infected urinary tract mucosa as illustrated in studies with IL-8 receptor knockout mice (221, 230).

The adaptive immunity does not play a major role in the early defense against UTI infection, since no difference with respect to resistance to infection was seen in lymphocyte deficient mice (nude, xid, scid, βTCR mutant, γTCR mutant, RAG-1 mutant) and their immunocompetent counterparts (231).

Dextran-sulphate (DX)-induced colitis mouse model

Unlike the urinary tract, the gut mucosa is constantly exposed to bacteria of the commensal flora. The bacterial species and number differ along the intestinal tract, with the highest population harboured in the colon (10^{11}-10^{12} bateria/g faeces in man) (232). The defense mechanisms at the mucosa of the gut face the difficult task to mount harmless responses to indigenous flora, while promptly counteracting the harmful antigens and pathogens. Unbalanced immune responses in the gut lead to inflammatory bowel diseases (IBD). IBD comprises two main clinical manifestations of gut inflammation, ulcerative colitis (UC) and Crohn´s disease. UC affects predominantly the large intestine and in Crohn´s disease any part of the gastrointestinal tract may be involved (233).

An experimental model, resembling features of human ulcerative colitis, induced by giving DX in the drinking water was described in mice, rat and hamsters (234-236). Giving DX to the animals over 7 days induces an acute colitis (234). DX is a sulphated polysaccharide, the polymer part consisting of repeating units of β1-6 glucose, and it is synthesized commercially from dextran produced by the lactobacillus *Leuconostoc mesenteroides* (237). A molecular weight of 30-40 kDa and a sulphur content of 15-17% were found to be optimal characteristics of DX for inducing colitis in mice (238). The histopathological changes induced by DX consist in epithelial exfoliation and recruitment of the inflammatory cells (macrophages, neutrophils, lymphocytes), with the involvement of the colonic mucosa, and in the later stages of the submucosa, where the oedema could be observed (239). The course of colitis was reported to be more severe in the endotoxin responsive mice at the later stage (240). The exact mechanism by which DX induces colitis is not completely elucidated. Direct cytotoxicity of DX on the intestinal epithelial cells, macrophage derived cytokines, and a macrophage impaired phagocytosis of bacteria were proposed to contribute to the pathogenesis of the model (239, 241, 242). Thus, macrophages were suggested to be crucial in the model. Macrophages containing DX were detected in the colonic mucosa, liver and mesenteric lymph nodes of animals, as early as after 1 day of DX exposure (243). An increased number of macrophages were detected by immunohistochemistry in the colonic mucosa, especially after seven days of DX exposure (244, 245). Elevated levels of macrophage derived cytokine mRNA and protein were reported in the colon (IL-1α, IL-1β, IL-6, TNF-α, IL-12), and draining lymph nodes (IL-1β, IL-6, TNF–α) of the animals exposed to DX (244, 246-248). The macrophage-induced cytokines may contribute to the tissue injury. IL-1β was shown to be important in the development of colitis, since the neutralization of this cytokine with antibodies improved the damages induced by DX (246). The T, B, NK and mast cells does not play a significant role in this model, since the colitis can be induced in SCID and T, B, NK and mast cell depleted mice (248-250).

The role of bacteria in the acute DX induced colitis is controversial, some studies showing the development of colitis in germ free animals, while an amelioration of the damages induced by DX is seen in studies using antibiotics (251-253). However, a shift in the normal colonic flora with increased numbers of *Clostridium* spp., *Bacteroidaceae* and *Enterobacteriaceae* is observed (234).

AIMS OF STUDIES

The major aim of the thesis was to investigate the anti-infectious and anti-inflammatory activities of LF and fragments thereof *in vivo*. In particular, studies were focused on:

- The anti-infectious effects of orally administered LF and two LF peptides, based on the anti-bacterial region of hLF molecule, on *E.coli* induced UTI in mice (I).

- The anti-inflammatory effects of orally given hLF on dextran-sulphate (DX)-induced acute colitis in mice (II).

- The mechanism for the inhibitory activity of LF on LPS-induced cytokine production in monocytic cell lines (III).

- Structural requirements for the microbicidal activities of the surface exposed helix-loop region of hLF (IV).

MATERIAL AND METHODS

LF and peptides

hLF and bLF from human milk and bovine colostrum respectively, were purchased from Sigma Aldrich (Stockholm, Sweden). The iron saturation of the LF batches used in the thesis was approximately 7%. The purity of LF was checked by SDS–PAGE electrophoresis.

The presence of the basic cluster of 4 arginine residues from the N-terminal domain of hLF corresponding to amino acids 2 to 5 (Arg^2-Arg^5) was checked by chromatography on Mono S column (254). The synthetic peptides used in the thesis were based on the antibacterial region of the hLF comprising an α helix and a β sheet (Fig. 5) and are shown in Tables 2 and 3 (I, IV). The hLF peptides were synthesized by a 9-fluorenyl-methoxy carbonyl continuos–flow strategy on a Biosearch Pioneer automated peptide synthethizer. Some peptides were capped at the N-(Ac) and C-(NH_2) terminal ends in order to neutralize the otherwise charged ends, which would not be present in the hLF molecule. HLD1 (referred to as HLBD1 in study IV) was made either linear or cyclic by introducing a disulphide bridge between the cystein residues. In HLD2 (referred to as HLBD2 in study IV) the cystein residues (C) were changed to acetamidomethylcystein (Cm) in order to avoid spontanous disulphide bridging. The peptides based on the helix region of hLF and the modifications made in the natural sequence are shown in Table 3.

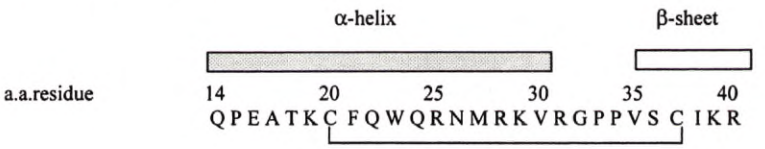

Fig. 5. Natural sequence of the antibacterial αβ helix region of hLF

Table 2. Peptides based on the antibacterial αβ region of hLF. The disulphide bridges between the cystein residues are indicated by lines.

Peptide code (MW)	sequence
HLD1 (3057 g/mol) or HLBD1	Ac - E A T K C F Q W Q R N M R K V R G P P V S C I K R – NH_2
HLBD1(Acm) (3201 g/mol)	Ac - E A T K C_mF Q W Q R N M R K V R G P P V S C_m I K R – NH_2
HLD2 (3002 g/mol) or HLBD2	Ac - T K C_mF Q W Q R N M R K V R G P P V S C_m I K R – NH_2
HLBD3 (3430 g/mol)	K C F Q W Q R N M R K V R G P P V S C I
HLBD3(Acm) (3576 g/mol)	K C_mF Q W Q R N M R K V R G P P V S C_mI

Table 3. Peptides based on the antibacterial α-helix region of hLF. The modifications made in the natural sequence are highlighted in bold. The lactam and disulphide bridges are indicated with lines.

Peptide code	sequence
Downsizing the helix region:	
HLBD4	Q P E A T K C F Q W Q R N M R K V R
HLBD5	P E A T K C F Q W Q R N M R K V R
HLBD6	E A T K C F Q W Q R N M R K V R
HLBD7	A T K C F Q W Q R N M R K V R
HLBD8	T K C F Q W Q R N M R K V R
HLBD9	K C F Q W Q R N M R K V R
HLBD10	C F Q W Q R N M R K V R
HLBD11	F Q W Q R N M R K V R
HLBD12	Q W Q R N M R K V R
Alanine scan:	
HLBDala1	**A** F Q W Q R N M R K V R
HLBDala2	C **A** Q W Q R N M R K V R
HLBDala3	C F **A** W Q R N M R K V R
HLBDala4	C F Q **A** Q R N M R K V R
HLBDala5	C F Q W **A** R N M R K V R
HLBDala6	C F Q W Q **A** N M R K V R
HLBDala7	C F Q W Q R **A** M R K V R
HLBDala8	C F Q W Q R N **A** R K V R
HLBDala9	C F Q W Q R N M **A** K V R
HLBDala10	C F Q W Q R N M R **A** V R
HLBDala11	C F Q W Q R N M R K **A** R
HLBDala12	C F Q W Q R N M R K V **A**
Substitution of the same kind of a.a.	
HLBDsk13	C F Q **L** Q R N M R K V R
HLBDsk14	C F Q W Q **K** N M R K V R
HLBDsk15	C F Q W Q R N **L** R K V R
HLBDsk16	C F Q W Q R N M **K** K V R
Negatively charged a.a.:	
HLBDE17	C F Q W **E** R N M R K V R
HLBDE18	C F Q W Q **E** N M R K V R
HLBDE19	C F Q W Q R **E** M R K V R
Charged or hydrophobic a.a.:	
HLBDsub20	C F Q W **Or** R N M R K V R
HLBDsub21	C F Q W **Nl** R N M R K V R
HLBDsub22	C F Q W Q R **Or** M R K V R
HLBDsub23	C F Q W Q R **Nl** M R K V R
Substitution:	
HLBD10opt1	C F Q W **K** R N M R K V R
HLBD10opt2	C F Q W **K** R **A** M R K V R
HLBD10opt3	C **F** **A** W **K** R N M R K V R
HLBD10opt4	C F **A** W Q R **A** M R K V R
HLBD10opt5	C F Q **L** Q **K** N M **K** K V R
HLBD10opt6	C **F** **A** **L** **K** **K** **A** M **K** K V R
Lactam bridges:	
HLBD1(18-22)2 (3069 g/mol)	Ac - E A ***E*** K C F ***K*** W Q R N M R K V R G P P V S C I K R - Amid
HLBD1(18-22)2 (3056 g/mol)	Ac - E A T K C F ***E*** W Q R ***K*** M R K V R G P P V S C I K R - Amid
HLBD1(18-22)2 (3085 g/mol)	Ac - E A T K C F Q W Q R ***E*** M R K ***K*** R G P P V S C I K R – Amid

The modifications of the natural sequence of antibacterial α helix region of hLF were obtained by replacing certain a.a.s with alanine (A), leucine (L), lysine (K), glutamic acid (E), ornithine (Or) and norleucine (Nl). In some peptides the lactam bridges were introduced at different positions between a.a.s E and K in order to induce some helix formation (Table 4). HLD1 cyclic and HLD2 linear peptides were used in the *in vivo* experiments (I, II), while both HLD1 cyclic and linear, HLD2, as well as the other peptides were used in *in vitro* studies (IV).

Limulus assay (III, IV)

The endotoxin contents of LF or peptides and the capacity of the peptides to neutralize LPS and lipid A were analysed by *Limulus* Amoebocyte Lysate assay (LAL) using a kit from Chromogenix. LF or peptides were solved in double distilled water (super Q) for endotoxin determination (III) or in Tris buffer (0.05M, pH 7.3) (IV). For the neutralizing activity determination, different concentrations of the peptides were incubated for 1 h at 37°C in pyrogen free tubes with 0.3 ng/ml LPS/lipid A. Two other LPS neutralizing agents (polymyxin B and a peptide based on the bactericidal/permeability increasing protein, BPI) were used for comparison (IV). The *Limulus* activity was analysed according to the manufacturer´s instructions.

Microorganisms (I, IV)

E.coli DS17 of the serotype O6:K5:H- was used in the UTI animal model (I). The strain originates from a child with acute pyelonephritis and expresses type 1 and P fimbriae and hemolysin. Bacterial strains *E. coli* O14, *E.coli* O6K5 (DS 17) *Klebsiella pneumonie* (CCUG 9997), *Enterococcus faecalis* (ATCC 19433); *Staphylococcus epidermidis* (CCUG 18000A), *Staphylococcus aureus* (CCUG 1800), and the yeast *Candida albicans* (ATCC 64549) were used in paper IV.

For UTI infection, the bacteria were cultured in Luria broth supplemented with 0.1% $CaCl_2$ at 37°C overnight or for two more days (I). For the microbicidal assay the microorganisms were cultured either as above (I) or all organisms were cultured in brain heart infusion medium (BHI) overnight at 37°C (IV). The bacteria were harvested by centrifugation, washed two times, diluted in PBS and adjusted spectrophotometrically to approximately 10^9 bacteria/ml (I, infection). For the microbicidal activity determination, a volume of the microorganism culture was transferred to a new tube with broth and incubated for two more hour at 37°C. Microorganisms were washed once and suspended in the broth used for microbicidal assay to a concentration of approximately $4x10^6$. The concentration of the bacteria was checked by viable counts.

Anti-microbial activity of LF or peptides (I, IV)

hLF, bLF or hLF peptides were serially diluted in medium. Different media were used depending on the experiment: BHI diluted 1:100 (I, IV), mouse urine (I) or 10 mM phosphate buffer pH 7.4 (I) or 1% bactopeptone (IV). The solutions (200 µl) were added to the microtiter plates (Nunclon, Nunc, Denmark). The microorganisms were added to the wells in a volume of 10 µl in order to give a final concentration of $2x10^5$/ml. The concentration of the

stock solution was always checked by viable counts. The plates were incubated at 37°C in a humid chamber for 2 hrs. Thereafter 5 µl was taken from each well and added as a drop onto a blood agar plate and the plate was incubated overnight at 37 °C. The concentration of LF or peptides giving 99% reduction of the inoculum was defined as MMC_{99}. In some experiments the viable count of microorganisms were determined for each concentration of LF or hLF peptides after 2 h of inoculation with the microorganism (I).

Protocols of oral administration of LF and LF peptides to mice (I, II)

hLF, bLF and the peptides HLD1 and HLD2 were given to the animals with *E. coli* induced urinary tract infection (UTI) (paper I). hLF and the two peptides were given to mice with DX-induced colitis. LF or peptides were dissolved in double distilled (super Q) water or PBS and given to mice perorally with a pipette in a volume of 50 µl on the back of the tongue. LF and peptides were given in a dose of 500 µg/mouse at 30 minutes after the instillation of bacteria into the urinary tract in the UTI model. The control groups received pure vehicle (PBS, water, or BSA solution).

Two different protocols of oral administration of hLF and peptides were used in the DX-induced model. They differed with respect to the time at which hLF were given to the animals in relation to the start of the DX-exposure. The dose of hLF or hLF peptides was the same in both protocols. hLF or peptides were given twice a day, once in the morning and once in the afternoon in a dose of 2 mg/mouse till the end of the experiments. hLF was given either prior to the DX exposure (no less than 30 min) or on the third day of DX-exposure. The DX exposed control group was given water or BSA. The animals were killed after 2, or 7 days of DX exposure.

Experimental UTI (I)

C3H/Tif and C3H/HeN female mice at least 8 weeks old (Charles River, Margate Trent, UK and Bomholtgård Breeding and Research Center Ltd. Ry, Denmark) were used.

Prior to inoculation the urinary bladders of mice were emptied by gentle compression of the abdomen. The urine from each mouse was collected and cultivated on lactose-bromthymol blue agar plates in order to check the sterility. The presence of PMN cells in the urine was checked by microscopy. The animals with bacteriuria or leukocyturia (>20 PMN/ml) were excluded.

Mice were infected under ether or methophane anesthesia by inoculation of 100 µl solution of 10^9 *E.coli* O6K5 bacteria/ml into the urinary bladder via a catheter (0.61 mm, Intramedic, Becton Dickinson, Sparks, Md.) attached to a 20-mm needle on a tuberculin syringe. Immediately after inoculation the catheter was gently withdrawn. The animals were killed 24 h after infection by cervical dislocation. The number of PMN in the mouse urine was determined 2, 6, and 24 h after the induction of UTI.

Determination of bacterial counts in organs

The number of viable bacteria present in the kidneys and urinary bladders of mice with UTI was determined by culturing suspensions of organ homogenates on blue agar plates. After killing of animals, the organs were removed aseptically and homogenized in 5 ml PBS in

disposable plastic bags. Serial 10-fold dilutions of 50 µl of organ homogenates were made in sterile PBS and 100 µl of each dilution were cultured on lactose-bromthymol blue agar plates. The plates were incubated overnight at 37°C. The bacterial concentration was expressed as colony forming units (CFU)/organ. The detection limit of the culturing was 100 bacteria per bladder or pair of kidneys.

E.coli ***adherence to mouse uroepithelial cells***

Uroepithelial cells from uninfected mice were collected by centrifugation (400xg for 10 min at room temperature) of urine pooled from 30 mice (C3H/Tif or C3H/HeN). The sediment was washed once and suspended in PBS to a concentration of 10^5cells/ml. A mixture of 200 µl of 10^9 CFU/ml of *E. coli* O6K5 bacteria suspended in PBS, 200 µl of uroepithelial cell suspension, and 100 µl of bLF, hLF, HLD1, HLD2 (10 mg/ml), or PBS was incubated on a rotator for 30 min at 37°C. After incubation, 3 ml of cold PBS were added and the cells were spun down, resuspended in PBS and fixed with one drop of neutral buffered formalin (Histofix; Histolab, Göteborg, Sweden). In one experiment with uroepithelial cells from C3H/HeN mice various concentrations of bLF (10, 5, 2.5 and 1,25 mg/ml) were first mixed with bacteria incubated on a rotator for 30 min at 37°C and thereafter with the uroepithelial cells. The mixture was incubated for 1 hr at 37°C with end over rotation and thereafter washed by repeated cycles of centrifugation and resuspension in PBS. The number of bacteria attached to at least 20 cells was determined. Adhesion was scored with an interference contrast microscope (Nicon Optiphot, at 500x magnification). The adherence value was defined as the mean number of adherent bacteria per cell.

Hemagglutination

The ability of LF and peptides to affect bacterial type P or type 1 fimbriae mediated hemagglutination of human or guinea pig erythrocytes, respectively was determined by hemagglutination assay.

Twenty five µl of twofold serially diluted solutions of bLF, hLF, HLD1 and HLD2 (10 mg/ml initial concentration) in PBS were mixed with 25 µl of *E. coli* O6K5 bacteria suspended in PBS at a concentration of 10^9 or $2x10^9$/ml in 96-well microtiter plates (U-shaped, Greiner, Laborteknik, Kebo, Sweden). The plates were shaken at room temperature (RT) for 30 min. Then, 25 µl of erythrocytes (washed three times and resuspended at a concentration of 2% in PBS) were added. The plate was shaken and incubated for 2 hrs at room temperature. Hemagglutination was read by eye and by bright-field microscopy at x40 magnification.

Bacterial agglutination

The ability of LF or peptides to agglutinate *E.coli* O6K5 bacteria through interaction with type 1 fimbriae was analized by an agglutination assay. Twenty five µl of bLF, hLF, HLD1, and HLD2 at a concentration of 10 mg/ml was serially diluted twofold in the microtiter plate. An equal volume of PBS or α-*methyl*-D-mannoside (25 mg/ml in PBS) and 50 µl of an *E. coli* O6K5 bacterial suspension ($1x10^9$) were added. The plate was shaken and incubated at 37°C for 30 min and then kept at 4°C overnight. The agglutination was read by eye and by bright-

field microscopy at x40 magnification. The reciprocal of the highest dilution rate giving bacterial agglutination was defined as the agglutination titer.

Dextran-sulphate-induced colitis in mice (II)

C57Bl/6J male mice at 8 weeks of age (Bomholtgård Breeding and Research Centre) were used.Acute colitis was induced by giving 5% dextran sulphate (DX) (TdB Consultancy, Uppsala) in the drinking water. DX with MW of 40-44 kDa and a sulphur content of 15-17% was used. Fresh DX solutions were prepared daily, and the pH was adjusted to 8.5. Animals were given DX for either 2 or 7 days. At the end of the experiments the animals were killed by cervical dislocation. The presence of occult blood was evaluated in the faeces of animals with DX-induced colitis by using a hemocult test (Bergman&Beving, Mölndal, Sweden). The animals were also inspected for macroscopic rectal bleeding.

Histology of colonic mucosa

The colon was removed, washed gently, divided into 3 segments (distal, middle and proximal) and each segment into two parts (one part was used for histology, one for immunohistochemistry, see below). The part for histology was fixed in 4% neutral buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin. The following scoring system was applied: score 0: intact crypt; score 1: loss of the basal third of the crypt; score 2: loss of the basal two thirds of the crypt; score 3: loss of the entire crypt with the surface epithelium remaining intact, score 4: loss of both the entire crypt and surface epithelium (239). The severity of colitis was estimated by measuring the areas with different scoring using a Leica microscope (Leitz, DMRB) connected to a computerized image-analysis system (Leica Q500 MC, Cambridge, UK). The evaluation was done blindly. The percentage of the damaged area to the total mucosa area was multiplied with its score and added together to a final score of the section.

Immunostaining and immunofluorescence (II, III)

Colon tissue (II)

Sections (5μm) of the frozen-Tissue Tek-embedded segments were made transversely using a cryostat (1720 Digital, Leitz), fixed in cold acetone (50% acetone for 30 seconds and then 100% for 5 minutes) and then air dried for 1 hr. The fixed sections were kept at –70°C in aluminium foil until stained. Depletion of endogenous peroxidase activity was performed on the sections for 20 minutes at room temperature with a solution of 1 U/l glucose oxidase (Sigma), 10 mM glucose, and 1 mM NaN_3. The sections were incubated for 15 minutes with avidin (Vector Labs, Burlingame, CA) followed by washing three times with PBS for 5 minutes and incubation for 15 minutes with biotin (Vector Labs) in order to block the endogenous avidin-biotin-binding-activity. After washing three times with PBS for 5 minutes, the slides were incubated overnight at 4°C with primary monoclonal antibodies diluted in PBS-Tween with 2% BSA and 2% goat serum (30 μl/section). The following monoclonal antibodies were used: F4/80 (F4/80 macrophage antigen, clone CI:A3-1, Serotec, Oxford, UK); L3T4 (CD4, clone RM4-5, Pharmingen, San Diego, USA); Ly-2 (CD8, clone 53-6.7, Pharmingen) and biotinylated mouse anti-mouse I-Ab (clone AF6-120.1, Pharmingen) diluted

at 5 μg/ml. Purified rat IgG2b (clone LO-DNP-11, Serotec) and IgG2a, κ, (clone P35–95, Pharmingen) in the same concentrations as the primary antibodies were used as isotype controls. Tissue sections were washed 3 times with PBS for 5 min between each step of incubation. For F4/80, CD4 and CD8 staining, the sections were incubated for one hour with the secondary biotinylated polyclonal goat anti-rat IgG antibodies (mouse IgG absorbed, Pharmingen, 2.5 μg/ml) diluted in PBS-Tween with 2% BSA. The slides were thereafter incubation with avidin-conjugated-peroxidase (ABComplex, Dako, Danmark) followed by with the peroxidase substrate solution consisting of 3-amino-9-ethyl-carbazole (Sigma) and H_2O_2 for 5 minutes. All sections were counter stained with Mayer´s hematoxylin for 5 minutes and mounted with Aquamount improved (BDH, Laboratory, Poole, England). The number of positively stained cells were evaluated by microscopy (Leitz, DMRB) connected to a computerised image-analysis system (Leica Q500 MC). The slides were analysed blindly. At least five randomly selected fields of each tissue segment were counted. F4/80 in submucosa, CD4 and CD8-positive cells were expressed as number of cells/area of tissue, while MHC class II ($I\text{-}A^b$) and F4/80 expression in the mucosa was recorded as the stained tissue area divided with the whole measured area x 100%.

The procedure for staining the cytokine positive cells in the colon tissues was similar as the protocol described above with some exceptions. The sections were fixed at 4°C for 8 min with a freshly prepared 2% paraformaldehyde in PBS. The endogenous peroxidase was blocked with 1% H_2O_2. Eagle buffered salt solution (EBSS) containing 0.1% saponin was used to prepare the washing buffer, the endogenous peroxidase and biotin blocking solutions, and to dilute the antibodies.

Saponin is a plant glycoside with high affinity for cholesterol and was used in order to allow the cytokine antibodies to penetrate through the cell surface membrane, the cytosol, and the membranes of the endoplasmic reticulum and the Golgi organelle. The permeabilization induced by saponin is reversible, therefore it is important to add it to all the incubation and washings steps. The following monoclonal antibodies were used for cytokine staining: rat anti-mouse TNF-α (IgG1, clone MP6-XT22, Pharmingen) and rat anti-mouse IL-10 (IgG 1, clone JES5-2A5, Serotec) diluted to a concentration of 5 μg/ml.

LF in monocytes (III)

The intracellular localization of exogenous LF in human monocytic cells was analyzed by immunostaining using LF specific antibodies. THP-1 cells were either cultured on chamber slides and treated for 4 hrs with hLF at 37°C, or the cells were settled in 24 well plates and treated with LF for different time points (10, 30, 240 min) at 37°C. The controls were treated with cell assay medium instead. The chamber slides were washed with PBS and allowed to dry for 30 min at room temperature. The cells collected from 24 well plates were washed by centrifugation three times with cold PBS, and the cell pellet rapidly frozen in liquid nitrogen as described for colon tissue. The chamber slides or 5 μm cryostat sections of the cell pellet were fixed in cold acetone and stained as described above for colonic tissue. For staining the chamber slides the following antibodies were used: as primary antibodies mouse monoclonal antibodies to hLF (IgG1 k) or isotype control mouse (Becton Dickinson) diluted at 8 μg/ml in PBS-T containing 2% BSA and as secondary antibodies biotinylated $F(ab')_2$ fragment of rabbit anti-mouse immunoglobulin (Dako, Denmark) diluted 1:400 in PBS-T containing

2%BSA and 2% normal serum. For staining the cryostat sections of the cell pellets the following antibodies were used: affinity purified goat anti-bLF (Bethyl Laboratory, Montgomery, TX) and rabbit anti-hLF immunoglobulin fraction (Dako) diluted 1:50 in PBS-T containing 2% HSA or BSA. The slides were analyzed by light microscopy.

To confirm the nuclear localization of LF, the confocal fluorescence microscopy was used. Cells were treated with LF at 37°C in 24 well plates. The cells were washed with cold PBS (3x). Thereafter, cytospin preparation of cells were obtained and fixed for 60 min with 2% paraformaldehyde in PBS. For preventing non-specific binding, PBS-T containing 2% BSA and 10% rabbit serum was added to the slides for 20 min. After washing, the slides were treated with 0.2% Triton X-100 in PBS for 2 min in order to permeabilize the cell membrane. Incubation with 2% BSA and 10% rabbit serum was repeated. After washing, the slides were incubated for 2 hrs with FITC conjugated affinity purified $F(ab')_2$ fragment of rabbit anti-human lactoferrin diluted 1:50 in PBS-T containing 2%BSA (hLF staining) or goat anti-bLF (affinity purified, Bethyl Laboratories) diluted 1:100 in PBS–T containing 2% HSA (bLF staining). For bLF staining, after washing, the slides were incubated for 1 h with rabbit $F(ab')_2$ anti-goat IgG–FITC labeled diluted 1:500 in PBS-T containing 2% HSA. The slides were washed in PBS, mounted in fluorescent mounting medium (Dako) and examined by a confocal microscope supplied with argon laser and equipped with an excitation filter set at 488, 568 and 647 nm (Carl Zeiss, Germany). A series of 15 optical sections at 1 μm intervals were recorded through cells.

ELISA (I, II, III)

The method was used in order to quantify the levels of hLF in the serum and urine of mice (I, II), the cytokines in the serum of mice with DX-induced colitis (II), and the cytokine secretion in the cell supernatants of LPS-stimulated human monocytic cell lines (III).

The basic protocol for hLF and human cytokines determination was similar, with some exceptions. The primary and secondary antibodies used in different ELISAs are indicated in Table 4.

The plates (Maxisorb, Nunc, Denmark) were coated overnight either at 4°C or room temperature with the primary antibodies appropriately diluted in the dilution buffer (0.1 M bicarbonate buffer pH 9.6 or PBS pH 7.4 for hLF and cytokine determination, respectively). After washing with PBS containing 0.05% Tween (PBS-T), the plates were blocked with the blocking buffer (5% fish gelatin in PBS or 1-5% BSA in PBS for hLF and cytokine determination, respectively) at room temperature for 1 or 16 hrs, depending on the protocol. For IL-10 and IL-12 determination the plates were left to dry at room temperature overnight after blocking. Thereafter, the samples and the standards appropriately diluted in the sample dilution medium (1% fish gelatine in PBS-T or cell assay medium consisting in RPMI 1640, 5% FCS and 1% pest) were added to the plates in duplicates. The biotinylated secondary antibodies diluted in 1% BSA in PBS were added together with the samples for the IL-10 and IL-12 determinations. The plates were incubated for 2 hrs at room temperature and then washed. For hLF determination alkaline phosphatase conjugated antibodies diluted in 1% fish gelatine in PBS-T were added. For cytokine determination (except IL-10 and IL-12), biotinylated secondary antibody appropriately diluted in 2% BSA in PBS were added. The

plates were incubated overnight at 4°C. After washing, the plates were incubated with the alkaline phosphatase substrate for hLF determination.

Table 4. Antibodies used in ELISA

antigen	primary antibodies	clone	company	conc. (μg/ml)	sample dil.	secondary antibodies	company	conc. (μg/ml)	detection enzyme	sensitivity of the assay	study
hLF	rabbit anti-hLF (IgG fraction)		Dako	18	undiluted 1:2	alkaline phosphatase conjugated rabbit anti-hLF	ICN	0.6		1.5 ng/ml	I,II
Human cytokines											
IL-1β	mono clonal mouse anti human IL-1β	6217.111	R&D Systems	4	1:2 1:4 1:20 1:40	biotinylated polyclonal goat anti human IL-1β	R&D Systems	0.1	alkaline phosphatase	16 pg/ml	III
TNF-α	mono clonal mouse anti human TNF-α	28401.111	R&D Systems	4	1:2 1:4	biotinylated polyclonal goat anti human TNF-α	R&D Systems	0.2	alkaline phosphatase	16 pg/ml	III
IL-6	mono clonal mouse anti human IL-6	6708.111	R&D Systems	4	1:2 1:4 1:20 1:40	biotinylated polyclonal goat anti human IL-6	R&D Systems	0.025	alkaline phosphatase	16 pg/ml	III
IL-8	mono clonal mouse anti human IL-8	6217.111	R&D Systems	4	1:25 1:50	biotinylated polyclonal goat anti human IL-8	R&D Systems	0.02	alkaline phosphatase	32 pg/ml	III
IL-10	mono clonal mouse anti human IL-10	B-N 10	Diaclone	1:100 dil	1:2	biotinylated monoclonal anti humaan IL-10	Diaclone	1:50 dil	horseradish peroxidase	16 pg/ml	III
IL-12	mono clonal mouse anti human IL-12	B-T 10	Diaclone	1:400 dil	1:2	biotinylated	Diaclone	1:50 dil	horseradish peroxidase	16 pg/ml	III

For cytokine detection, the plates were incubated for 20 minutes with either horseradish peroxidase conjugated streptavidin (diluted 1:6700 in 1% PBS-T, IL-10 and IL-12) or alkaline phosphatase conjugated extravidin diluted 1:1000. After washing 4 times, the substrates for alkaline or horse radish peroxidase, respectively (1 mg/ml p-nitrophenyl phosphate in 10 mmol/l diethanolamine, 0.5 mmol $MgCl_2$, pH 9.5 or tetramethylbenzidine) were added to the plates. The color development was either read in a microplate reader (Multiskan Bichromatic, Labsystem, Göteborg, Sweden) at 405 nm or the reaction was first stopped with 1 M H_2SO_4 and then read at 450 nm.

Mouse serum samples were analysed for IL-1β, TNF-α, IL-6, IL-10 and IL-12 using ELISA kits (R&D system and Biosource International, Camarillo, CA) according to the manufacturer's protocols. Serum samples were diluted 1:2. The detection limit for these cytokines were 8 pg/ml for IL-1 β, 15 pg/ml for IL-12, 30 pg/ml for IL-6 and IL-10, and 47 pg/ml for TNF-α.

IL-6 determination by bioassay (I, III)

The method was used to measure IL-6 levels in urine and serum of animals with UTI, and IL-6 secretion in the supernatants of LPS-stimulated THP-1 cells (I, III). B9 hybridoma cell line which is dependent on IL-6 for growth was used. The cells were harvested from the tissue flasks and seeded into microtiter plates at the concentration of 5000 cells/well. Samples or IL-6 standard (Genzyme Corporation Cambrige, MA) were added to the cells and incubated for 72 hrs in Iscove's modified Dulbecco's medium supplemented with $5x10^{-5}$ M β-mercaptoethanol and 5% fetal calf serum (FCS). ^{3}H-thymidine was added 4 hrs before harvesting the cells. One unit of IL-6 per ml was required for half-maximal proliferation of B9 cells (approximately equivalent to 1 pg).

Cell assay (III)

The ability of LF to affect the LPS-induced cytokine production was investigated in two human monocytic cell lines (THP-1 and Mono Mac 6) which were stimulated with *E. coli* LPS (O55:B5). THP-1 was obtained from ATCC (Manassas,VA, USA) and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (GIBCO, BRL, Life Technologies INC , Gaitesburg, MD), 1% sodium pyruvate (Gibco) and 2.5% HEPES (Gibco). Mono Mac 6 was obtained from DSM (Braunschweig, Germany) and maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1% sodium pyruvate, 1% L-glutamine, 1% bovine insuline and 1% non essential amino sugars (Gibco). The cells were maintained at 37°C in a 5% CO_2 incubator. The two cell lines differed with respect to the maturity. THP-1 cells are less matured, expressing low levels of CD14 and MHC class II on the cell surface, while Mono Mac 6 cells are more matured, expressing higher levels of CD14 and MHC class II. The THP-1 cells were prestimulated for 16 hrs with 100 U/ml IFNγ (human recombinant, produced in *E. coli* >99% pure, endotoxin level<10EU/1x10^5U, Boehring Mannheim Biochemica, Mannheim, Germany) in order to increase the sensitivity to LPS. For the cell experiments, the cell density was adjusted to 1x10^6cells/ml in the cell assay medium (RPMI 1640 containing 5% FCS and 1% pest) and 400 µl were added to 24 well-

plates. The cells were allowed to settle for 1 hr in the cell incubator and thereafter stimulated with 5-10 ng/ml of LPS (*E. coli* serotype O55:B5; Sigma Chemical CO., St. Louis, Mo) for different time points (0, 1, 2, 4, 6, 12 and 18 hrs). Thirty minutes before or after the LPS addition, the cells were given LF. In one experiment, the cells were stimulated with 250 ng/ml recombinant IL-1β (rIL-1b, R&D systems, Abingdon, UK). The viability of the cells in the presence of LPS or/and LF ranged within 85-95% as measured by trypan blue exclusion.

Reverse transcriptase PCR (RT-PCR) (III)

The cytokine mRNA expression was analysed by a semiquantitative RT-PCR. A simultaneuosly amplified sequence of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used for comparison. The total RNA was extracted from the cell pellets with a ready to use reagent, which contained phenol and guanidinium thiocyanate in a monophase solution (RNA STAT-60, Tel-Test"B" Inc.; Friendswood, TX, USA). After being extracted with chloroform, precipitated with isopropanol and washed with ethanol according to the manufacturer´s instructions, the RNA was treated with RNase-free DNaseI (Promega, Falkenberg, Sweden), in order to remove the genomic DNA. The yield and purity of RNA was quantified by measuring the ratio of the optical density at 260 and 280 nm.
Successful isolation of undegraded RNA was monitored by minigel electrophoresis in 3% agarose in the presence of ethidium bromide and identification of the 28S and 18S ribosomal RNA bands.
One μg of extracted RNA was used for cDNA synthesis in a 30 μl reaction mix containing 3.3 mM random hexamer primer (pd(N)6; Amersham Pharmacia Biotech, Sweden), 1xRT buffer (GIBCO) 0.5 mM dNTP (dATP, dCTP, dGTP and dTTP; Amersham Pharmacia Biotech), 1U/ml human placenta RNAase inhibitor (Promega) and 13.3U/ml reverse transcriptase (Superscript;GIBCO), and diethyl pyrocarbonate–treated water (DEPEC-H_2O). The reaction was carried out at 42°C for 60 min. After synthesis, samples were stored at -70°C until the PCR amplification. For each cytokine, commercially available specific primers, positive and negative controls (Clontech Laboratories, Palo Alto, CA, USA) were used for PCR amplification. The housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was simultaneously amplified. Commercial available primer pairs for cytokines were used. Two μl of cDNA were added to a reaction mixure with a final concentration of 1X PCR II (Perkin Elmer), 2mM of $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each primer and 1.25U Taq polymerase (AmpliTaq gold; Perkin Elmer) in a final volume of 50 μl. The PCR profile used was denaturation at 94°C for 45s, annealing at 60°C for 45s, and extension at 72°C for 2 min (Gene Amp PCR system 9600, Perkin Elmer). The gene products were separated by electrophoresis in 3% agarose gel containing ethidium bromide. The gels were photographed in UV light by a polaroid camera. IPLab Gel software was used for densitometric analysis of the bands of the scanned gel images. The levels of cytokine mRNA were expressed as the ratio between the cytokine and the G3PDH gene products for each sample.

Preparation of nuclear protein extracts (III)

Cells were harvested on ice after 1 or 2 hrs of stimulation with LPS and washed two times by centrifugation (300g, 5 min) at 4°C with cold PBS. Hypotonic buffer (1.5 ml containing 10

mmol/l HEPES pH 7.9, 0.1mmol/l EGTA, 10mmol/KCl, 2.5mg/ml BSA, 0.75mmol/spermidine, Sigma, 0.15 mmol/l spermine, Sigma, Complete ™ proteinase inhibitors, Roche Diagnosics, Mannheim, Germany, and 1 mmol/l DTT, Roche Diagnostics) was added to the cell pellet. The cells were homogenized on ice by 8 strokes with a B pestle in a Dounce homogenizer. Cell nuclei were collected by centrifugation at 16000 *g* at 4°C for 10 min. The nuclei were resuspended in 60-100 µl of elution buffer (20 mmol/l HEPES pH 7.9, 25% glycerol 0.42 mol/l NaCl, 1 mmol/l EDTA,1mM/l EGTA, 1mmol/DTT) supplemented with the same proteinase inhibitors as in the hypotonic buffer. After extraction for 45 min at 4°C, the nuclear proteins were separated from nuclei by centrifugation at 16000 *g* at 4°C for 1 h. The supernatant containing the proteins, was collected and stored in aliquots at –70°C. Protein concentrations of the nuclear extracts were measured according to Bradford using a protein assay kit (Pierce, Stockholm, Sweden). Bovine serum albumin was used as a protein standard.

Electrophoretic mobility shift assay (EMSA) (III)

The ability of LF to interfere with NF-κB binding to the TNF–α promoter was analyzed by EMSA. Nuclear protein extracts were analyzed by EMSA for their ability to bind to a specific α ^{32}P labeled oligonucleotide, corresponding to the sequence –646 to -613 on the human TNF-α promoter containing a binding site for the transcription factor NF-κB. The protein–DNA binding reaction was carried out in a volume of 20 µl containing 20 mM Tris-HCl, pH 7.9, 75 mM NaCl, 0.1 mM EGTA, 1mM DTT, 5% glycerol and 5 µg of poly (dI-dC)poly (dI-dC), (Amersham Pharmacia Biotech, Sweden). Approximately 70,000 Cerenkov cpm corresponding to 2 ng of the probe and equal amounts of nuclear protein extracts were added to each reaction mixture and allowed to proceed for 30 min at room temperature. The reaction mixtures were then separated by electrophoresis through a native 5% polyacrylamide gel containing 0.25X TBE (2.2 mmol/l tris borate, 2.2 mmmol/l boric acid, 0.5 mmol/l EDTA) and 3% glycerol, to distinguish DNA-protein complexes from unbound DNA probe.

Statistics

Differences between groups were analysed for significance by either non parametric Kruskal Wallis test followed by Dunn´s post test for multiple comparison (II), or ANOVA test followed by Bonferoni post test for multiple comparison (III) when more that 2 groups were compared. Mann-Whitney *U*-test was used when two groups were compared (II). This test was also used in I. Frequencies were analyzed by Fisher´s Exact test (II). Differences were considered significant for values of $p<0.05$.

RESULTS AND DISCUSSION

Orally given hLF and peptides based on the antibacterial region of hLF are active against experimental *E.coli* UTI in mice (I)

The effect of orally given LF and peptides based on the anti-bacterial region of hLF (HLD1 and HLD2) on the infection and inflammation induced at a remote site from the gut, the urinary tract, was investigated in this study.

LF or peptide treatment was initiated 30 min after the induction of UTI. Since *in vitro* studies showed a more potent antibacterial effect of bLF than hLF, two mouse strains were used in the first series of experiments for bLF treatment (C3H/Tif and C3H/HeN) for comparing the effects in similar, but not identical strains. The hLF effect was assessed only in C3H/Tif mice. The number of bacteria present in the kidney and urinary bladder 24 hrs after infection was significantly reduced in LF treated animals compared to the control. Twenty nine and fifty seven percent of the animals in the hLF treated group had no detectable bacteria in the kidneys and bladder respectively, but none in the control group (Figs. 1 a and 2 a, I). The corresponding figures for the bLF group were 14 and 35 percent respectively, in the C3H/Tif mice and 11 and 39 in the C3H/HeN mice (Figs 1 and 2, I). The inflammatory parameters (number of PMN and IL-6 levels in the urine as well as IL-6 in the serum) were also reduced in the LF treated animals compared to the controls (Figs. 3, 4, and 5, I).

Since the overall results of the first series of experiments showed that hLF was somewhat more efficient than bLF in reducing the infection and inflammation in the urinary tract, synthetic peptides based on the antibacterial region of hLF were compared with hLF in the next series of experiments. The results showed that the linear peptide HLD2 was as effective as hLF in reducing the number of bacteria in the kidney (Table 2, I). A similar pattern was seen for HLD1 although protection was not quite significant (Table 2, I).

A summary of the main effects of LF and peptides on the parameters analyzed is shown in Table 5.

The next question asked was if hLF is taken up into the circulation and transported to the urinary tract, exerting its activity locally. The analysis of hLF in the serum by ELISA showed that it was present in the serum of 11 out of 24 animals treated with hLF, but not in those treated with the vehicle, indicating that the protein can pass over to the circulation (data not shown). Moreover, by ELISA, hLF was found in the urine of uninfected mice given hLF (Fig. 5). Thus, hLF effects could be mediated locally.

Next, the possible mechanism of the hLF effects in the urinary tract was addressed. Since adherence of bacteria to the uroepithelial cells is the first step in the pathogenesis of UTI, the interference of LF with this activity was investigated. Agglutination of bacteria by bLF has been reported and thus this aspect was also investigated. hLF or the peptides affected neither adherence nor agglutination of bacteria. However, the mannosylated bLF blocked the adherence of *E.coli* by 50% and bacterial agglutination via binding to type 1 fimbriae (data not shown). This indicates that the interference with mucosal adherence of bacteria is not the mechanism responsible for the anti-infectious effects of hLF or peptides.

Table 5. The effects of LF or peptide treatment on the parameters analyzed in UTI compared to the control.

	No. of bacteria present in the urinary tract 24 hrs after inoculation		No of PMN in urine			IL-6 levels in urine			IL-6 levels in serum
treatment	kidneys	bladder	2	6	24 hrs	2	6	24 hrs	24 hrs
hLF	↓[a]	↓[b,c]	↓	(↓)	↓[h]	↓	**no**	**no**	↓
HLD1	↓[d]	**no**	n.d.	n.d.	**no**	n.d.	n.d.	n.d.	n.d.
HLD2	(↓)[e]	**no**	n.d.	n.d.	**no**	n.d.	n.d.	n.d	n.d.
bLF	↓[f]	↓[g]	↓[i]	**no**	**no**	↓[j]	**no**	**no**	**no**

↓, significantly reduced parameter; (↓), reduced, but not significant; **no**, parameter not changed; n.d. not done; [a] 29 and 25% of animals free of detectable bacteria in the first and second series of experiments, respectively; [b] 57% of the animals free of detectable bacteria in the first series of experiments ; [c] not significantly reduced in the second series of experiments, however the inflammatory response of the host was reduced in these experiments compared to the first series; [d] 17% of the animals free of detectable bacteria; [e] 16% of the animals free of detectable bacteria; [f] 14 and 11% of animals free from detectable bacteria in the C3H/Tif and C3H/HeN groups, respectively ; [g] 35 and 39% of the animals free of detectable bacteria in C3H/Tif and C3H/HeN, respectively; [h] not significant in the second series of experiments; [i] only reduced in C3H/Tif, but not in C3H/HeN mice; [j] reduced only in C3H/Tif, but not in C3H/HeN mice.

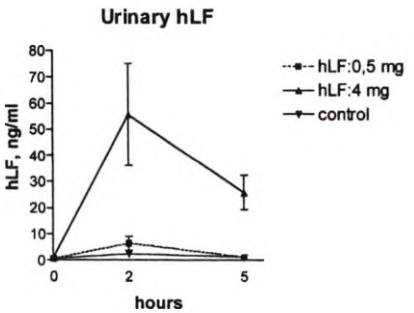

Fig. 5. Levels of hLF in the urine of C3H/Tif mice treated perorally with hLF. The bars represent the mean and standard error of urinary hLF levels in four to six animals per time point.

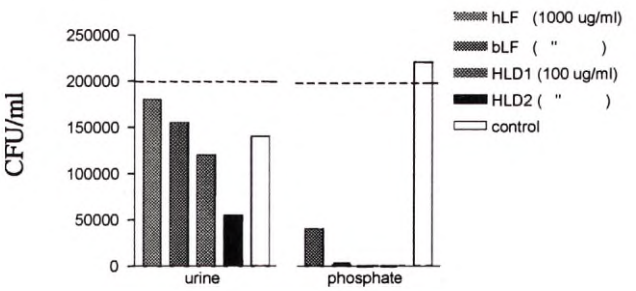

Fig. 6. Concentration of E.coli *O6K5 bacteria in undiluted urine and phosphate buffer in the presence of hLF, bLF, HLD1 or HLD2 after 2 hrs of incubation The concentration of bacteria in the inoculum was $2x10^5$ CFU/ml and the number of bacteria in the controls were $1.4x10^5$ and $2.2x10^5$ CFU/ml.*

A 60% reduction of the bacterial number was observed in undiluted urine in the presence of HLD2 compared to the control after 2 hrs of incubation (Fig. 6). Urine by itself had a bacteriostatic and bactericidal effect on bacteria. Both HLD1 and HLD2 killed 100% of the inoculum in the phosphate buffer (Fig. 6). hLF and bLF reduced the concentration of bacteria to 18 and 2% respectively.

The results indicated that hLF is effective in reducing infection and inflammation in the urinary tract. The effects are possibly mediated by the hLF or peptides locally. The bactericidal activity of hLF, or fragments thereof is suggested as a possible mechanism. The bactericidal activity of the peptides are affected by the salt concentration. Therefore, it is possible that the milieu of the renal tubuli with a low salt concentration might be the site were the peptides are the most effective against bacteria. Whether the intact hLF molecule or fragments thereof is the main component reaching the circulation has not been elucidated. It is not excluded that the mechanism by which hLF exerts its effects may be multifactorial. Since anti-bacterial and anti-inflammatory activities have been described for hLF *in vitro* it is possible that both of these properties also act *in vivo* (163). Thus, the anti-inflammatory activity could be either a consequence of the anti-bacterial effect or the downregulation of IL-6, or both.

Anti-inflammatory activities of hLF in acute DX-induced colitis in mice (II)

The anti-inflammatory activities of hLF *in vivo* were investigated using a model of inflammation of the gut. Acute colitis was induced in mice by giving DX in the drinking water for either 2, or 7 days. Oral administration of hLF and peptides was initiated either before or after an inflammation had been established , e.g., on the third day of the DX exposure. In the control group the hLF or peptide solution was replaced by solely vehicle. The appearance of blood in the faeces was delayed and partly reduced in the hLF treated animals (Figs. 1 a, b, and 2 a, b, II). The shortening of the colon, a pathological effect of the DX exposure, was reduced in the hLF treated animals compared to the control (Figs. 1 c. Table 2, II). Treatment with hLF starting on the third day of the DX exposure also resulted in

diminished bleeding and contraction of the colon. Also the IL-1β levels in the blood were significantly diminished in the hLF treated group compared to the control (Fig. 1 d, II). A significantly reduced crypt score and reduced numbers of $CD4^+$, F4/80 positive macrophages, and TNF-α producing cells were detected by histology and immunohistochemistry in the distal colon of hLF treated animals after 7 days of DX exposure (Fig. 2 c, II, and Fig.7). The HLD1 and HLD2 gave almost identical results to those of hLF concerning the clinical parameters after two days of DX exposure (Fig. 1, II). A summary of the effects of orally given hLF or peptides in the acute colitis is presented in Table 6.

Table 6. The effects of hLF or peptide treatment on the clinical parameters in DX induced colitis compared to the control.

DX exposure (days)	treatment	occult blood	macroscopical rectal bleeding	colon length	serum IL-1β	crypt score distal colon
2	hLF	(↓)	(↓)	(↑)	↓	**no**[a]
	HLD1	(↓)	(↓)	↑	↓	**no**[a]
	HLD2	↓	(↓)	↑	↓	**no**[a]
7	hLF	↓*	↓**	n.d	**no**	↓
	hLF[b]	n.d.	↓	↑	n.d.	n.d.

↓ significantly reduced parameter; (↓), reduced, but not significant; **no**, parameter not changed; n.d. not done; [a] no significant damage in the colon after 2 days of DX exposure; [b] hLF treatment started on the third day of DX exposure; * fewer hemocult positive mice during the first 3 days of DX exposure, and significant only on day 2 of DX exposure **fewer mice with macroscopical rectal bleeding on days 3, 4 and 6 of DX exposure, and significantly fewer on day 4.

hLF was detected in the serum of animals perorally treated with hLF, but not in the controls (Table 1, II), indicating that the protein can reach the colon not only via the gut, but also through the circulation. The histopathological damages induced by DX in the colon consisted in epithelial exfoliation and infiltration of inflammatory cells. The damages induced by DX were more pronounced after seven days in the distal and middle parts of the colon, while the proximal part was less affected. The effects of hLF on the length of the colon and the improved crypt score could be due to its reported ability to stimulate the proliferation of epithelial cells (255). Although the role of bacteria in the initiation of DX-induced colitis model is debated, there is a shift in the normal bacterial flora (251, 252). Thus, increased numbers of the *Clostridium* species, *Bacteroidaceae* and *Enterobacteriaceae* were detected in the faeces of mice exposed to DX (234). This may have some bearings on the development of the disease. A contribution of bacteria and bacterial products in the severity of the later phase of the DX induced colitis was reported (240).

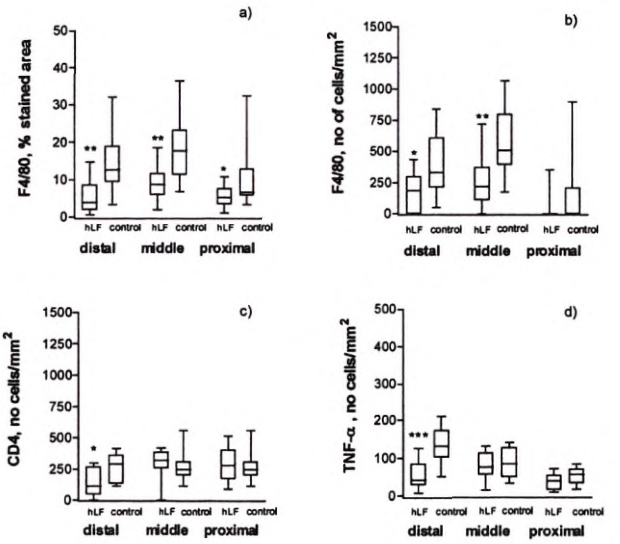

*Fig. 7. The number of F4/80-positive cells in the colonic mucosa (a) and submucosa (b), the number of CD4-positive cells (c), and TNF-a producing cells (d) in the colonic mucosa of mice treated with DX for seven days. The DX-treated mice were given hLF or water (controls). The boxes indicate the 25th, 50th and 75th percentiles and the bars the lowest and the highest values. *p< 0.05, ** p<0.01, Mann Whitney U test.*

Antibacterial activities against *Clostridium* spp. were reported for orally given bLF *in vivo* in a model of colonization of mice with these bacteria (127). Thus, it is possible that the antibacterial activities of hLF or peptides against members of *Clostridium* and *Enterobacteria* (*E. coli*) could have contributed to the reduced symptoms observed in the hLF or peptide treated mice compared to the controls.

The macrophages are suggested to contribute to the pathogenesis of the DX-induced colitis (234, 248). An increased number of these cells (F4/80 positive) was detected in our study in the colonic mucosa and the submucosal oedema after seven days of DX exposure compared to DX-unexposed animals (Fig. 3 a, II and data not shown). The increased number of F4/80 macrophages in the colonic mucosa has also been reported by others (244). The macrophages may secrete cytokines which contribute to the tissue damage (244-247). A lower number of F4/80 positive macrophages was found in the middle part of the colon of the hLF- and HLD1 treated groups after 2 days of DX exposure compared to the control (Fig. 1 e, II). The hLF treatment also significantly reduced the number of infiltrating F4/80 macrophages in the colonic mucosa and submucosa after seven days of exposure (Fig. 7 a, b). This finding correlated with a reduced number of TNF-α producing cells in the distal colonic mucosa (Fig. 7 d) and IL-10 producing cells in the submucosa oedema of the middle part of the colon of the hLF treated mice compared with controls (data not shown).

hLF treatment reduced the number of CD4-positive cells, either by a indirect or direct mechanism. Althougth the CD 4 positive cells are not crucial for the induction of DX colitis,

the reduction by LF treatment may reflect a reduced immune response. *In vitro* studies have shown that hLF could inhibit proliferation and cytokine production of a CD4/TH1, but not of a TH2 cell line. This effect could posibly have some bearing on the observed reduction of CD4 positive cells.

In conclusion, hLF has anti-inflammatory activities in the colon and its bactericidal region may contribute to these activities.

Lactoferrin down-regulates the LPS-induced cytokine production in monocytic cells via NF-κB (III)

Our *in vivo* studies showed that hLF is mediating anti-inflammatory activities in the colon and urinary tract (I, II). LPS induced host effector mechanisms have reported to contribute to the severity of the disease in the later phase of DX induced colitis (240). LPS is a potent inducer of inflammation in the urinary tract (223). *In vitro* studies have demonstrated that LF down-regulates the LPS-induced IL-1β, TNF-α and IL-6 cytokine production in monocytic cells (145, 163, 164). The mechanism by which LF is inhibiting the LPS-induced cytokine production in THP-1 and Mono Mac 6 monocytic cell lines was studied in paper III, by investigating the effect of LF on cytokine mRNA expression and the involvement of NF-κB.

LF inhibited the LPS-induced cytokine production in a monocytic cell lines in a dose dependent manner and regardless of the time at which LF was added to the cells in relation to LPS (30 min before or after) (Fig. 1, III and Figs 8, 9).

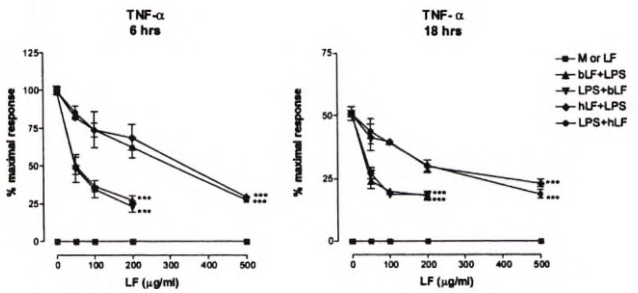

*Fig. 8. Dose-inhibitory activity of LF on LPS-induced TNF-α secretion in Mono Mac 6 cells. bLF (50, 100, 200 μg/10⁶ cells) or hLF (50, 100, 200, 500 μg/10⁶ cells) were added to the cells 30 minutes before (bLF+LPS or hLF+LPS), or after (LPS+bLF or LPS+hLF) the addition of LPS (5 ng/10⁶ cells). The results are expressed as percentage of maximal response. The mean±SEM from three separate experiments are indicated. ***p<0.01, ANOVA test followed by Bonferoni test for multiple comparison. The statistical significance for the highest dose of LF versus control is indicated.*

LF inhibited the LPS-induced secretion of TNF-α to 82-85% of the response, IL-6 to 56-68%, IL-1β to 44-64%, and IL-8 to 20-34 % in THP-1 cells at 18 hrs (Table 1, III). This inhibitory effect was obtained with a dose of 50 and 200 μg/ml for bLF and hLF. Increasing the concentration to 200 and 500 ug/ml for bLF, and hLF respectively, did not significantly

increase the inhibitory effect.

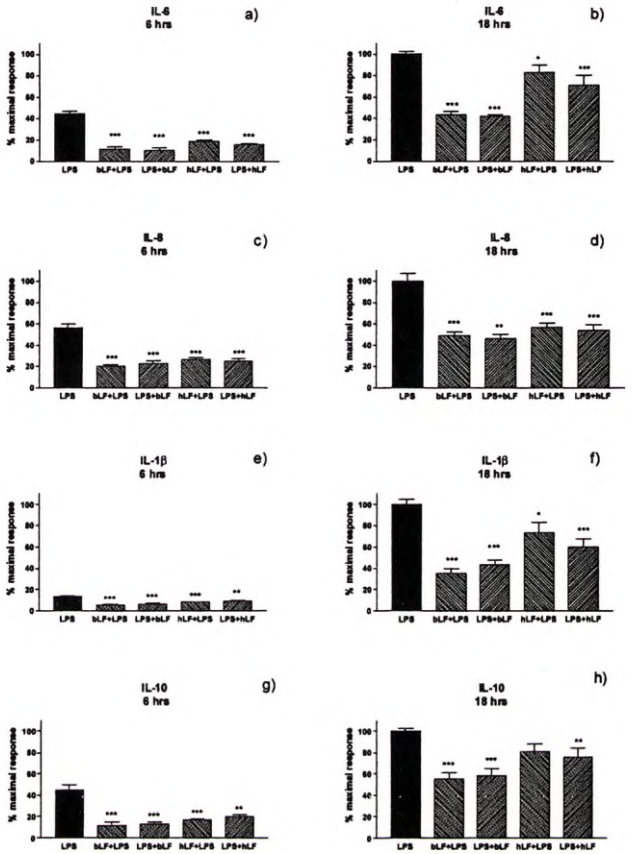

*Fig. 9. The effect of LF on IL-6 (a,b), IL-8 (c, d), IL-1β(e, f) and IL-10 (g, h) secretion in the supernatants of Mono mac 6 cells. Cells were given bLF (200 μg/10^6 cells) or hLF (500 μg/10^6 cells) 30 minutes before (bLF+LPS or hLF+LPS) or after (LPS+bLF or LPS+hLF) the addition of LPS (5 ng/10^6 cells). The results are expressed as percentage of the maximal response. The means±SEM from three separate experiments are indicated. ***p< 0.001.*

The effect of LF on cytokine secretion was similar when monitored in a more mature monocytic cell line, Mono Mac 6 (Figs. 8, 9). The dose of LF needed to inhibit the LPS-induced TNF-α secretion to 70-80% was 200 and 500 μg/ml for bLF and hLF, respectively (Fig. 8). At these doses LF also inhibited the IL-6, IL-8, IL-1β and IL-10 after 6 and 18 hrs.

Since the cytokines are mostly regulated at the transcriptional level, the effect of LF on cytokine mRNA expression was further investigated in THP-1 cells. By a semiquantitative RT-PCR method a lower level of LPS-induced TNF-α, IL-1β, IL-6 and IL-8 mRNA was

detected at the peak of expression in THP-1 cells treated with bLF (Fig. 10).

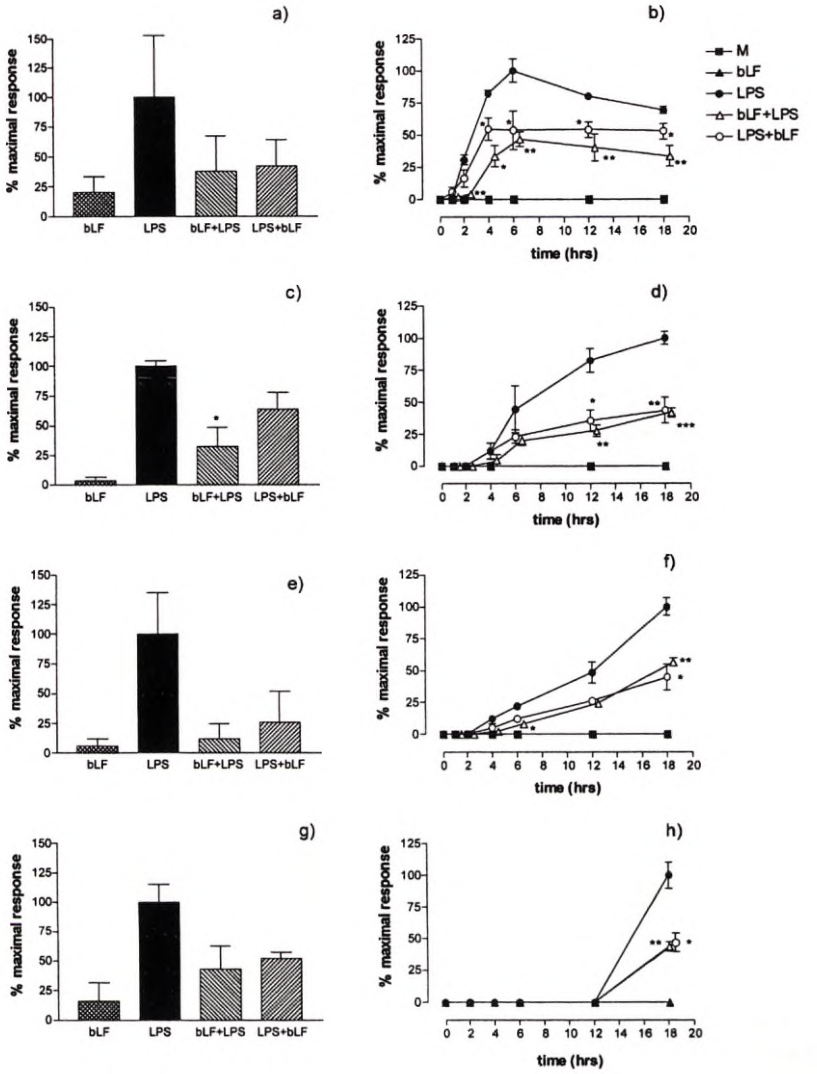

*Fig. 10. The effect of bLF on LPS-induced mRNA expression (a, c, e, g) and the kinetics of cytokine secretion (b, d, f, h) in THP-1 cells . Cells were given bLF (50 μg/10 ^{6}) 30 minutes before or after the addition of LPS (10 ng/10 ^{6} cells). The TNF–α (a), IL-6 (b), IL-8 (e) and IL-1β(g) mRNA expression was assesed by RT-PCR using G3PDH as a house keeping gene. The effects of bLF at the peak of the cytokine mRNA expression are presented (2 hrs in a, e, and g, 4 hrs in c). The results are expressed as a percentage of maximal response. The mean±SEM from three separate experiments are indicated. *p<0.05, **p<0.01 , ***p<0.001.*

The reported binding of LF to LPS and the prevention of LPS binding to CD14 receptor via competition with LBP could partly explain the inhibitory activity on cytokine production (105, 106, 109). However, inhibition was seen in our system both when LF was added to the cells before and after LPS. Moreover, it was previously shown that LF inhibits the TNF-α induced IL-6 secretion and in this study we noted the ability of hLF to inhibit the IL-1β-induced IL-6 secretion (163). These results suggested that LF may have a more general effect on cytokine transduction pathways. Several studies have shown that LF is taken up by monocytic cells (150, 167-169). However, the fate of the protein and the effects intracellularly are not fully elucidated (150, 169, 256). Therefore, we followed the uptake of LF intracellularly by immunohistochemistry and fluorescence confocal microscopy. LF was detected in THP-1 cells as early as after 10 min of incubation and in the nucleoli after 30 min (Figs. 3, 4 and Table 3 in III).

Since NF-κB regulates the transcription of pro-inflammatory cytokine genes, the interference of LF with LPS induced activation of this transcription factor was further investigated. A decreased binding of NF-κB to the human TNF-α promoter was found by EMSA after 1 h of incubation with LPS when LF was added to the cells 30 minutes before LPS (Fig. 5 a, III). The response was dose dependent, a dose of 500 and 200, but not 50 μg/10^6 cells of hLF decreased the DNA binding of NF-κB. The decreased LPS-induced binding of NF-κB by LF was also seen after 2 hrs of incubation with LPS irrespective of the time at which LF was added to the cells in relation to LPS (Fig. 11). LF by itself had no effect on the binding of NF-κB to DNA.

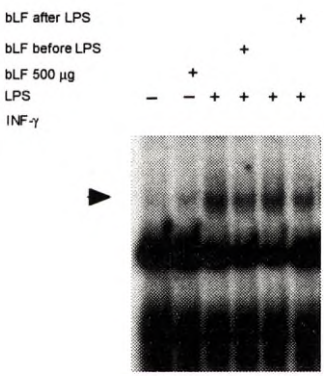

Fig. 11. The effect of bLF on the LPS induced binding of NF-κB to oligonuleotides from human TNF-α promoter. Nuclear extracts from THP-1 cells untreated or treated with LPS for 2 hrs in the presence or absence of LF were analyzed by EMSA for binding to α ^{32}P labeled oligonuleotide from TNF-α promoter –646 -613.

The results suggest that LF inhibits the pathway leading to NF-κB translocation to the nucleus or has a more direct effect on the NF-κB in the nucleus. Competition between NF-κB and LF for binding to the TNF-α promoter is excluded, since no binding of LF to the oligonucleotides

was found by EMSA. The decreased binding of NF-κB to the TNF-α promoter with increased concentration of LF suggested that LF may bind to NF-κB.

The fact that LF, within 2 hrs, down-regulated the LPS-induced cytokine gene expression by inhibition of NF-κB to DNA indicates that no new gene expression is necessary for this inhibition. In summary, LF inhibits the LPS-induced cytokine mRNA via interference with NF-κB.

Structure-activity relationship of the αβ antibacterial region of hLF (IV)

The hLF derived peptides used in the experimental UTI and colitis models were based on the antibacterial domain located in the N-terminal end in an αβ region (Fig 5). Peptides based on this region were synthesized and the structure-microbicidal activity relationship was investigated *in vitro* in study IV. The importance of the disulphide bridge (position 20-37) and helix like formation was analysed with respect to the antimicrobial activity. The length and role of single amino acids of the helix region required for expressing optimal microbicidal activity were assessed. Microbicidal analyses (99% killing) were performed by a microplate assay.

The cyclization via the disulphide bridge in HLBD1 (a.a residues 16-40) and HLBD3 (a.a. residues 19-38) increased somewhat the microbicidal activity against *E.coli* and *S.epidermidis*, but HLBD3 against *S.aureus* only (Table 1, IV). Downsizing of the peptide sequence corresponding to residues 14-31 starting from the N-terminal end showed that four sequences (a.a. residues 17-31, 18-31, 19-31, 20-31) were the overall most active peptides as analysed against 7 bacterial strains and *C. albicans* (Table 2, IV). An alanine-scan of the a.a. residues 20-31 (HLBD10), showed that replacement of cysteine (C)-20 and tryptophan (W)-23 reduced the killing activity against the three analysed microorganisms (*E.coli*, *S. aureus*, and *C. albicans*) (Table 7). Replacing the arginine (R) residues at the end of HLBD10 (positions 28 or 31) corresponding to the amphiphatic helical region also affected the killing activity, mostly against *E.coli* and *C.albicans*. Changing, however, W or R to the same type of a.a. (leucine, L or lysine, K) did not reduce the killing activity (Table 5 in IV). As expected, increasing the hydrophobic or charged a.a. content of HLBD10 improved its activity.

The kinetics of the microbicidal activity of HLBD1 and a modified a.a. sequence of HLBD10 (HLBD10opt3, Table 3) showed that the longer a.a. sequence killed *C.albicans* within 10 min, *S.aureus* within 1 h, and *E.coli* after 2 hrs (Fig 12 a). A somewhat different pattern was seen with the small peptide regarding *S.aureus* which was killed within 30 min (Fig 12 b).

The differences in the kinetics of killing are probably due to the different cell envelopes of the three microorganisms. *E. coli* has an outer membrane, containing LPS, proteins and phospholipids not present in *S. aureus*. The cell envelope of *C. albicans* contains mannoproteins and polysaccharides. The cytoplasmic membrane of *C. albicans* is also different by containing ergosterol. Theoretically, it would be expected to take more time for a peptide to penetrate two membranes as in *E. coli*.

One of the mechanisms for the killing of *E. coli* by Lfcin has been suggested to be mediated via the binding of the peptide to LPS. Therefore, the most potent peptides were studied for their neutralization of the *Limulus* activity of LPS and lipid A.

Table 7. Microbicidal activity of the peptides derived from HLBD10 (a.a. 20-31) (CFQWQRNMRKVR) by alanine scan. The peptides were incubated for 2 h with E.coli, S. aureus, and C. albicans *in diluted BHI medium (BHI_{dil}) or 1% bactopeptone (BP). The concentration of peptides needed for 99% killing is shown (MMC_{99}).*

position	a.a. replaced by alanine	MMC_{99}, µg/ml					
		E.coli		*S. aureus*		*C. albicans*	
		BHI_{dil}	BP	BHI_{dil}	BP	BHI_{dil}	BP
20	C	> 25*	>100#	14	>100	12	>100
21	F	12	n.d.	7	n.d.	12	n.d.
22	Q	6	50	3.5	100	6	25
23	W	25	>100	14	>100	12	>100
24	Q	12	50	3.5	100	12	50
25	R	12	n.d.	7	n.d.	12	n.d.
26	N	6	25	3.5	50	6	25
27	M	12	50	3.5	>100	25	50
28	R	25	>100	7	>100	25	>100
29	K	12	100	3.5	>100	25	> 100
30	V	12	50	3.5	>100	25	50
31	R	12	>100	7	>100	25	>100
	none	12	100	7	>100	12	50

The small peptides corresponding to a.a. 17-31, 18-31, 19-31, and 20-31 (HLBD7-10) inhibited the *Limulus* activity of lipid A from 60 to 90%. (Fig. 13 a). Using the longer peptides HLBD1 and HLBD2, the neutralization effect was at most 30% for the diphosphoryl lipid A. The peptide HLBD9 (a.a. residues 19-31) reduced the *Limulus* activity of O111 LPS to 54 and 055 LPS to 46% (Fig. 13 b). This inhibitory effect was comparable to the LPS neutralization agents polymyxin B and BPI.

In summary, this study showed that a 13 a.a. long fragment comprising more than half of the antibacterial α helix region in the hLF molecule was the most active microbicidal fragment against *E. coli, S. aureus* and *C. albicans*, but the 12, 14, and 15 a.a. long fragments were of similar activity. The C residue at the N terminal end (position 20), a hydrophobic residue at position 23 and the charged a.a. at positions 28 and 31 were important for the antimicrobial activity. The 12-15 a.a. long peptides may contribute to the killing of *E. coli* by binding to LPS/lipid A, thereby damaging the integrity of the outer membrane.

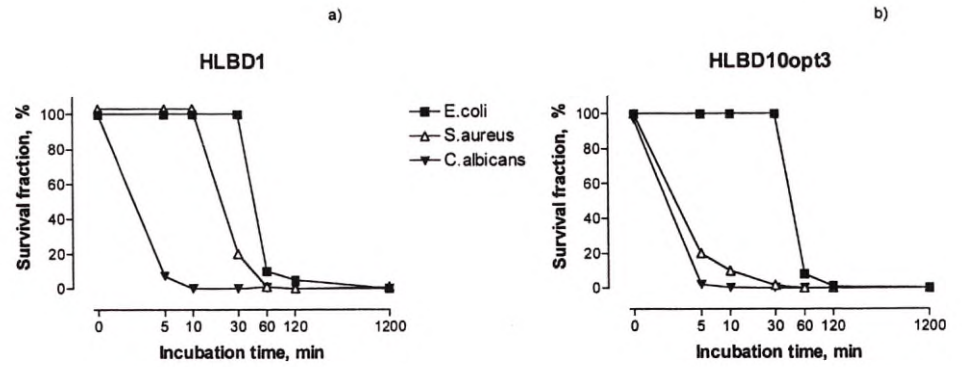

Fig. 12. Kinetics of the killing activity of the peptides HLBD1 (a) and HLBD 10 opt3 (b). The peptides were incubated with E.coli *O6K5,* S. aureus (1800), and C. albicans *in BP for 20 hrs using 200 μg/ml of HLBD1 and 100 μg/ml of HLBD10 opt3. Samples were collected directly after mixing and at 5, 10, 30, 60, 120 min and 20 hrs. Each point represents the percentage of the inoculum remaining alive.*

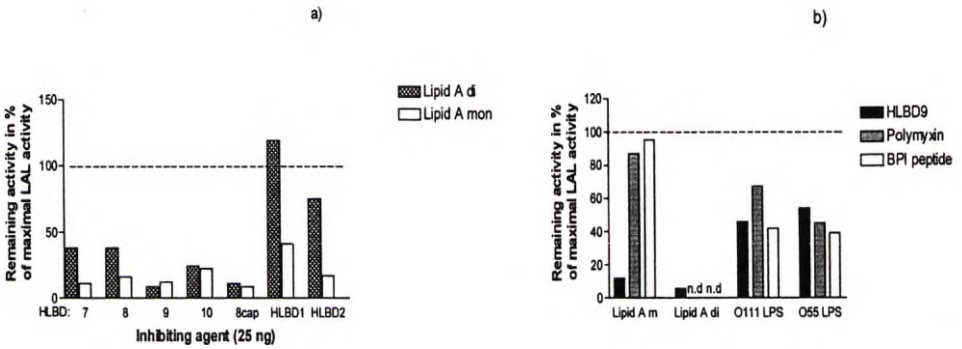

Fig. 13. Neutralization of the Limulus *activity of di- and monophosphoryl lipid A (300 pg/ml) (a) by incubation with HLBD peptides (25 ng/ml); and LPS (O111 and O55, 300 pg/ml) (b) by incubation with HLBD9, polymyxin B , and BPI peptide (5 μg/ml). (n.d. means not determined).*

CONCLUDING REMARKS, HYPOTHESES AND EMERGING QUESTIONS

The innate immunity at mucosal surfaces plays a crucial role in protection against infection and in development and modulation of inflammatory responses. The evolutionary conserved factors provided by the secretions at mucosal sites constitute key elements in the first line of defense (257). They consist of antimicrobial proteins and peptides that are either constitutively expressed or induced in response to infection and inflammation (257). Their role in defense is usually a result of the ability to act as antimicrobials, as well as to orchestrate the cells of the immune system in the response (257). The role of lactoferrin as a factor of innate defense is supported by its presence in high concentration in the mucosal secretions, and its antimicrobial and immunomodulatory effects, noted in *in vitro* studies.

Since the immune system of the newborn is not fully developed and functional, the support provided by the mother for protection at mucosal surfaces is crucial because this is where most infections are initiated especially in children (258). It is well documented that breastfeeding provides protection against respiratory and urinary tract infections, diarrhea, necrotizing enterocolitis, otitis media and septicemia (258, 259). There is also evidence that the enhanced protection against certain infections, including UTI can last after the termination of breast feeding (258, 260). The defense factors present in milk offer such a protection. The most studied component in this respect are the sIgA antibodies which are directed against microbes that the mother has encountered in her gut previously in life (258).

The role of orally given LF in protection against infection and inflammation at mucosal membranes has been poorly explored *in vivo*. Therefore, the major question addressed in this thesis was if anti-infectious and anti-inflammatory activities at the mucosal surfaces could be mediated by the orally given LF. The mechanism by which LF down regulates cytokine production in monocytes was also addressed by *in vitro* studies. Our results clearly demonstrated that orally given LF is effective *in vivo* in reducing infection and inflammation at a remote site, the urinary tract and mediates anti-inflammatory activities in the colon. The dual effects of hLF may at least partly reside in the antimicrobial region of the molecule, since we also found synthetic fragments based on this region to be effective *in vivo*. One of the possible mechanisms by which LF mediates its anti-inflammatory effects *in vivo* could be its ability to down regulate pro-inflammatory cytokines at the transcriptional level by interference with the NF-κB transcription factor, as we have shown *in vitro*.

The prerequisite for orally given LF or fragments to exert its biological activities locally at mucosal surfaces such as in the urinary tract or the colon, is their ability to reach these sites. Using the ELISA method we detected hLF in the serum and urine of animals fed hLF, indicating that hLF pass via the circulation and in this way can reach the urinary tract, and in addition to the oral route, also the colon. However, to which extent LF or the synthetic peptides are degraded during the passage through the intestinal tract was not elucidated in our study. Our attempts to identify by electrophoresis and immunoblotting the hLF or its fragments in the urine of animals with *E. coli* urinary tract infection, which received orally hLF, were not successful. This was due to the fact that the anti-hLF antibodies cross-reacted with the mLF present in the urine of the experimental animals in response to the infection,

resulting either from the urinary PMN or possibly from secretions. Using a double sandwich ELISA, we detected hLF in the urine of uninfected animals fed hLF. However, we do not exclude that larger fragments possibly generated by degradation of hLF during the passage through the intestinal tract may be a major part in this system of detection. The peptides HLD1 and HLD2 did not react in our ELISA method (data not shown). We have also tried to detect the peptides HLD1 and HLD2 in the urine of infected animals by mass spectrometry and purification on chromatography using beads reported to bind cationic peptides. However, the lower sensitivity of the mass spectrometry (>100 ng) and the interference with the host defensins (261), present in urine in response to infection and having approximately the same molecular weight as HLD1 and HLD2 peptides, made our attempts unsuccessful. The labeling of the peptides would be preferable. However, this might very well change the peptide conformation, uptake, and possibly binding to the intestinal receptor for hLF. Receptors for LF on the brush borders of the small intestine of rhesus monkey and human infant intestine, with preference for binding hLF and monkey LF, but not bLF have been reported (262). The number of binding sites for the receptor of hLF is higher on the intestine of the suckling than older infants (75). Beside the entire hLF molecule, the large fragments corresponding to the N terminal lobe of the hLF molecule also bind to the receptor (75). In mice, the intestinal receptor binds LF from different species with the same affinity (69, 70). The role of these receptors in the uptake of LF in the circulation is not known. LF and large LF fragments have been detected by others in the urine and faeces of human infants fed human milk (63, 65, 66, 263).

To which extent the microbicidal fragment LFcin or even smaller fragments are generated *in vivo* by digestion of LF during its passage through the gastrointestinal tract in humans, especially in infants, and in mice is little investigated. In our studies, the synthetic hLF fragments were as effective as hLF in reducing the infection and inflammation in the urinary tract and the inflammation in the colon. Smaller fragments (12 –15 a.a long) than those used *in vivo* (23-25 a.a. long) were similar or sometimes even better in their killing activity against *E. coli*, *S. aureus* and *C. albicans in vitro*. If these fragments are also generated *in vivo*, as a result of degradation of LF or the synthetic fragments in the intestine or at the site of infection remains to be determined. It is of interest to explore to which extent active LF fragments are generated *in vivo*, especially in the suckling newborns, who have a lower secretion of gastric acids and synthesis of digestive enzymes than older children and adults (264). Would these fragments be formed *in vivo* by enzymatic digestion with matrix proteases that are present at mucosal sites in inflammatory conditions, or by proteases secreted by bacteria?

A lower risk of infections in the gut and urinary tract has been observed in breast fed infants (258, 259). Our results of an anti-infectious and anti-inflammatory effect of hLF or synthetic fragments in the urinary tract may help explain such an observation. Thus, hLF may provide, as the sIgA antibodies much of the mucosal protection transferred by the mother via the milk to the offspring.

hLF or LF fragments may possibly interact with other components of the host defense in regulating the antimicrobial activity at mucosal surfaces. A synergy between LF and sIgA or lysozyme has been described *in vitro* (96-98, 265).

The suckling neonate could benefit from the dual antimicrobial and anti-inflammatory property of LF or its fragments, especially during the colonization and expansion of the intestinal microflora. It is known that there are differences with respect to the predominating species in the intestine of breast fed compared to bottle fed infants. Thus, breast fed infants have lower counts of clostridia, a tendency to a lower count of enterobacteria, and higher counts of staphylococci than bottle fed infants (266-268). hLF together with sIgA may contribute to the colonization pattern of the gut by limiting the number of colonizers and protecting against pathogen invaders in the period when the infant still has a somewhat immature immune system of its own.

Our results also showed the ability of LF to down-regulate LPS-induced pro-inflammatory cytokines in monocytic cells. An activation of the cellular innate system of the infant by the new colonizers carrying LPS and resulting in pro-inflammatory cytokines, could be down regulated by hLF. TNF–α was found to be the most down regulated cytokine by LF in our *in vitro* system.

TNF-α is a potent pro-inflammatory cytokine and induces the expression of adhesion molecules on the endothelial cells, fever, the secretion of other pro-inflammatory cytokines such as IL-1 and IL-6, and is thus an important mediator of inflammation, septic shock and certain inflammatory diseases e.g. rheumatoid arthritis (138). The down regulation of this early pro-inflammatory cytokine by LF in the gut may prevent a strong inflammation with consequences for the integrity of the gut mucosa.

In our *in vitro* system we noted the down regulation by LF of also TNF-α and IL-1β–induced cytokines. We suggest that one possible mechanism for the down-regulation of the proinflammatory cytokines is through interference with their common gene regulator, the transcription factor NF-κB. This finding may have potential therapeutic use. NF-κB is known to be important in the pathogenesis of many diseases such as IBD, rheumatoid arthritis, diabetus, and thus a therapeutic target (269).

Since LF was found intracellularly in monocytic cells, we do not exclude, however, and it remains to be determined, if it may interfere with other signal transduction pathways leading to proinflammatory cytokines, such as MAPKases. The exact mechanism of LF interference with NF-κB needs also to be elucidated. Whether LF binds to NF-κB in the nucleus, if the possible NF-κB-LF complex is transported to the nucleoli where we find LF in the cells, are open questions.

In addition to increasing our knowledge about the role of LF as an important element of the innate defense at mucosal surfaces, the anti-infectious and anti-inflammatory activities of LF and fragments thereof may possibly be of potential therapeutic value.

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